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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

Allelic frequencies for the seventeen Y-STR loci observed in Iraqi male patients with prostate cancer

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Prostate cancer is a significant disease in men and a large number of individuals would benefit if risk factors that increase the susceptibility to develop this neoplasia could be established, which could aid in the early detection of the disease that is crucial for successful treatment. The first objective of this study was detection of allelic frequencies of 17 Y-chromosome short tandem repeat loci from Iraqian prostate cancer patients and normal control males. The second objective was evaluating the association of these loci with the presence of prostate cancer. Blood samples were collected from 100 unrelated male patients living in Middle and South of Iraq. FTA® Technology was utilized to extract DNA from blood collected on FTA™ paper. One 1.2 mm punch from a card containing whole blood was loaded into the appropriate wells of the reaction plate. The PCR was realized with the GeneAmp® PCR System 9700 thermal cycler. Post PCR amplification was detected using an ABI Prism1 3130xl Genetic Analyzer 16-capillary array system, with POP-7™ Polymer and Data Collection Software, GeneMapper version 3.5. Six alleles (20 to 25) for DYS635, three alleles (14 to 16) for DYS437, five alleles (18 to 22) for DYS448, five alleles (13 to 17) for DYS456, seven alleles (14 to 20) for DYS458, five alleles (10 to 14) for YGATA H4, three alleles (12 to 14) for DYS389I, six alleles (28 to 33) for DYS389II, five alleles (13 to 17) for DYS19, five alleles (8 to 12) for DYS391, four alleles (9 to 12) for DYS438, five alleles (21 to 25) for DYS390, six alleles (9 to 14) for DYS439, four alleles (11 to 14) for DYS392, three alleles (13 to 15) for DYS393, eight alleles (11 to 19) for DYS385a and eight alleles (13 to 20) for DYS385b were found among the whole Iraqi subjects examined. A higher incidence of disease was found among males who had either allele 10 of DYS391 or allele 13 of DYS393. It is likely that Iraqi males who belong to Y-lineages with either allele 10 or allele 13 are more susceptible to develop prostate cancer, while those belonging to lineages with allele 9 and 14 of DYS439 or allele 15 of DYS385b are more resistant to the disease. This study shows the influence of genetic-factors on prostate cancer, and it seems that DYS391 and DYS390 loci comprising DYS635, DYS437, DYS448, DYS456, DYS458, YGATA H4, DYS389I, DYS389II, DYS19, DYS438, DYS439, DYS392, DYS393, DYS385a and DYS385b STRs have the potential to be used as a screening method for prediction of susceptibility to prostate cancer in Iraqi population.

Key words: Allele frequency, FTA™ paper, Iraq, prostate cancer, STR DNA typing, Y filer™.

INTRODUCTION

Microsatellites are a group of molecular markers chosen for a number of purposes including forensics individual identification and relatedness testing polymorphism (Kimpton et al., 1996; Gill et al., 2001; Andrea et al., 2008). There is a high genomic abundance of random distribution throughout the genome; also abundance of

polymorphism (Ellegren, 2004; Butler and Hill, 2012). The Y-chromosome is specific to the male portion of a male-female DNA mixed such as is common in sexual assault cases. These STRs can also be useful in missing person's investigations, historical investigations, some paternity testing scenarios, and genetic genealogy (Kwak et al., 2005).

Although, they are often used to suggest which haplogroup an individual matches, STR analysis typically provides a person haplotype. Most tests on the Y chromosome examine between 12 and 67 STR markers (Carolina et al., 2010; Mohammed and Imad, 2013; Muhanned et al., 2015a). The Y chromosome is less variable than the other chromosomes. Many markers are thus needed to obtain a high degree of discrimination between unrelated males' marker.

Prostate cancer is a significant disease in men accounting for approximately 33% of all male cancers and having a 9% mortality rate for men presenting with disease (Jemal et al., 2006). However, public awareness for prevention and early detection of prostate cancer is relatively low. Two classifications are used to describe prostate cancer. The Union for International Cancer Control (UICC) 2002 classifies it as tumour, node and metastasis (TNM) which is commonly used for malignant tumours (Hayes et al., 2005). The second classification system, Gleason score, is specific for grading of adeno-carcinoma of the prostate (Gleason and Mellinger, 1974).

A large number of individuals would benefit if risk factors that increase the susceptibility to develop prostate cancer could be established, which could aid in the early detection of the disease which is crucial for successful treatment (Paracchini et al., 2003; Ewis et al., 2006; Mohammed et al., 2015; Ameera et al., 2015). Numerous studies have been conducted on the molecular genetic aetiology of the disease. The incidence of prostate cancer varies considerably between people of various ethnicities (Parkin et al., 1993; Hsing et al., 2000; Jemal et al., 2006; Muhanned et al., 2015b), which suggests that in part the predisposition for developing prostate cancer is associated with alleles that are more prevalent in certain populations or groups.

In this study, we discuss the role of 17 susceptibility genes commonly debated within the field of prostate cancer research.

MATERIALS AND METHODS

Preparation of blood samples

Blood samples were collected from two hundred unrelated males patients living in middle and south of Iraq. Comprising 100 men with prostate cancer and 100 healthy male individuals as control. All patients participated in the study were males over 40 years old who had been referred to the hospital for treatment because of advanced level of cancer.

DNA extraction and amplification

DNA was extracted from all dried blood samples on FTA cards following the manufacturer's procedure as described in Whatman FTA Protocol BD01 except that the Whatman FTA purification reagent was modified to half the volume. A 1.2 mm diameter disc was punched from each FTA card with a puncher. The discs were transferred to new Eppendorf tubes and washed 3 times in 100 µl Whatman FTA purification reagent. Each wash was incubated for 5 min at room temperature with moderate manual mixing and the reagent was discarded between washing steps. The discs were then washed twice in 200 µl TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), the buffer was discarded and the discs were left to dry at room temperature for 1 h. A commercial kit Y filer™ PCR amplification kit (Applied Biosystems, Foster City, CA) that amplifies 17 Y-STR loci: DYS635, DYS437, DYS448, DYS456, DYS458, YGATA H4, DYS389I, DYS389II, DYS19, DYS391, DYS438, DYS390, DYS439, DYS392, DYS393, DYS385a and DYS385b and a segment of the amelogenin gene was used, according to manufacturer's instructions but in a total reaction volume of 25 µl. Amplification for Y-chromosomal STR regions were carried out using sets of primers (Table 1).

The master mix was homogenized by vortex for 3 s, centrifuged briefly, then 25 µl of PCR amplification mix was pipetted into each reaction well. One 1.2 mm punch from a card containing whole blood was loaded into the appropriate wells of the reaction plate. The positive amplification control, 1 µl of 2800 M control DNA (10 ng/µl) was added to a reaction well containing 25 µl of PCR amplification mix.

The protocol used with the GeneAmp® PCR System 9700 thermal cycler is provided below. PCR program is as follows: 96°C for 1 min, then: 94°C for 10 s, 59°C for 1 min, 72°C for 30 s, for 25 cycles, then: 60°C for 20 min and soaked at 4°C. After completion of the thermal cycling protocol, the amplified samples were kept or stored at -20°C in a light-protected box.

The amplicons were visualized using the ABI Prism1 3130*x*/ Genetic Analyzer 16-capillary array system (Applied Biosystems, Foster City, CA, USA) following manufacturer's protocols, with POP-7™ Polymer and Data Collection Software, GeneMapper version 3.5 software (Applied Biosystems). The allele designations were determined by comparison of the PCR products with those of allelic ladders provided with the kit. Nomenclature of loci and alleles is according to the International Society of Forensic Genetics (ISFG) guidelines reported in Gill et al. (2001). By comparison of the size of a sample's alleles to size of alleles in allelic ladders for the same loci being tested in the sample, the STR genotyping was conducted.

Statistical analysis

A. Allele diversity was calculated as (Nei, 1987).

$$D = \frac{n}{n-1}\left(1 - \sum_{i=1}^{n} p_i^2\right)$$

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Abbreviation: ISFG, International society of forensic genetics.

Table 1. Primer sequence, forward (F) and reverse (R) for DYS genetic loci.

DYS loci	Primer sequence (5' - 3') (Forward; F, Reverse; R)
YGATA H4	F: 5'- ATGCTGAGGAGAATTTCCAA -3' R:5'- GCTATTCATCCATCTAATCTATCCATT -3'
DYS19	F: 5'- ACTACTGAGTTTCTGTTATAGTGTTTTT -3' R: 5'- GTCAATCTCTGCACCTGGAAAT -3'
DYS385a	F: 5'- AGCATGGGTGACAGAGCTA -3' R: 5'- GCCAATTACATAGTCCTCCTTTC -3'
DYS389I	F: 5'- CCAACTCTCATCTGTATTATCTATG -3' R: 5'- GTTATCCCTGAGTAGTAGAAGAATG -3'
DYS389II	F: 5'- CCAACTCTCATCTGTATTATCTATG -3' R: 5'- GTTATCCCTGAGTAGTAGAAGAATG -3'
DYS390	F: 5'- CCAACTCTCATCTGTATTATCTATG -3' R: 5'- GTTATCCCTGAGTAGTAGAAGAATG -3'
DYS391	F: 5'- TTCATCATACACCCATATCTGTC -3' R: 5'- GATAGAGGGATAGGTAGGCAGGC -3'
DYS392	F: 5'- TAGAGGCAGTCATCGCAGTG -3' R: 5'- GACCTACCAATCCCATTCCTT -3'
DYS393	F: 5'- GTGGTCTTCTACTTGTGTCAATAC -3' R: 5'- GAACTCAAGTCCAAAAAATGAGG -3'
DYS438	F: 5'- CCAAAATTAGTGGGGAATAGTTG -3' R: 5'- GATCACCCAGGGTCTGGAGTT -3'
DYS439	F: 5'- TCGAGTTGTTATGGTTTTAGGTCT -3' R: 5'- GTGGCTTGGAATTCTTTTACCC -3'
DYS635	F: 5'- ACCAGCCCAAATATCCATCA -3' R: 5'- TGGAATGCTCTCTTGGCTTC -3'
DYS437	F: 5'- GACTATGGGCGTGAGTGCAT -3' R: 5'- GAGACCCTGTCATTCACAGATGA -3'
DYS448	F: 5'- TGGGAGAGGCAAGGATCCAA -3' R: 5'- GTCATATTTCTGGCCGGTCTGG -3'
DYS456	F: 5'- GAGGAATCTGACACCTCTGACA -3' R: 5'- GTCCATATCATCTATCCTCTGCCTA -3'
DYS458	F: 5'- GCAACAGGAATGAAACTCCAAT -3' R: 5'- GTTCTGGCATTACAAGCATGAG -3'

Where, *n* is the sample size and *pi* is the frequency of the *i*th allele.

B. Standard error (SE): The standard error (SE) of allele frequencies was calculated as:

$$SE(p_i) = \sqrt{[(1 - p_i)\, p_i]/N},$$

Where, *pi* denotes the frequency of the *ith* allele at any given locus and N equals the total number of individuals screened at this locus.

RESULTS AND DISCUSSION

Based on Y-chromosome database (http://www.smgf.org, Sorenson Molecular Genealogy foundation), DYS19 is STR consisting 10 alleles with 7 to 15 repeats of TAGA motif, DYS385a/b is STR consisting 22 alleles with 7 to 23 repeats of GAAA motif, DYS389I is STR consisting 9 alleles with 6 to 13 repeats of TCTG motif, DYS389II is

Table 2. Allelic frequencies of (YGATA H4, DYS19, DYS385a, DYS385b, DYS389I, DYS389II, DYS390, DYS391 and DYS392) genetic loci observed in 100 Iraqi males patients with prostate cancer.

Alleles	YGATA H4		DYS19		DYS385a		DYS385b		DYS389I		DYS389II		DYS390		DYS391		DYS392	
	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	lociS.E.
8	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.09	0.03	·	·
9	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.06	0.02	·	·
10	0.15	0.04	·	·	·	·	·	·	·	·	·	·	·	·	0.66	0.08	·	·
11	0.23	0.05	·	·	0.13	0.03	·	·	·	·	·	·	·	·	0.13	0.03	0.58	0.08
12	0.41	0.06	·	·	0.03	0.02	·	·	0.15	0.04	·	·	·	·	0.06	0.02	0.16	0.04
13	0.1	0.03	0.28	0.05	0.47	0.07	0.07	0.03	0.39	0.06	·	·	·	·	·	·	0.16	0.04
14	0.11	0.03	0.11	0.03	0.09	0.03	0.31	0.06	0.46	0.07	·	·	·	·	·	·	0.1	0.03
15	·	·	0.45	0.07	0.04	0.02	0.02	0.01	·	·	·	·	·	·	·	·	·	·
16	·	·	0.11	0.03	0.09	0.03	0.1	0.03	·	·	·	·	·	·	·	·	·	·
17	·	·	0.05	0.02	0.05	0.02	0.15	0.04	·	·	·	·	·	·	·	·	·	·
18	·	·	·	·	0.1	0.03	0.1	0.03	·	·	·	·	·	·	·	·	·	·
19	·	·	·	·	0.13	0.03	0.2	0.04	·	·	·	·	·	·	·	·	·	·
20	·	·	·	·		·	0.05	0.02	·	·	·	·	·	·	·	·	·	·
21	·	·	·	·		·	·	·	·	·	·	·	0.12	0.03	·	·	·	·
22	·	·	·	·		·	·	·	·	·	·	·	0.21	0.04	·	·	·	·
23	·	·	·	·		·	·	·	·	·	·	·	0.06	0.02	·	·	·	·
24	·	·	·	·		·	·	·	·	·	·	·	0.48	0.07	·	·	·	·
25	·	·	·	·		·	·	·	·	·	·	·	0.23	0.04	·	·	·	·
28	·	·	·	·		·	·	·	·	·	0.1	0.03	·	·	·	·	·	·
29	·	·	·	·		·	·	·	·	·	0.41	0.07	·	·	·	·	·	·
30	·	·	·	·		·	·	·	·	·	0.28	0.05	·	·	·	·	·	·
31	·	·	·	·		·	·	·	·	·	0.13	0.03	·	·	·	·	·	·
32	·	·	·	·		·	·	·	·	·	0.02	0.01	·	·	·	·	·	·
33	·	·	·	·		·	·	·	·	·	0.06	0.02	·	·	·	·	·	·
D		0.991		0.99		0.971		0.99		0.98		0.99		0.991		0.991		0.989

S.E., Standard error; F., allelic frequencies.

STR consisting 11 alleles with 24 to 34 repeats of TCTG motif, DYS390 is STR consisting 12 alleles with 17 to 28 repeats of TCTG motif, DYS391 is STR consisting 9 alleles with 6 to 14 repeats of TCTA motif, DYS392 is STR consisting 11 alleles with 6 to 17 repeats of TAT motif, DYS393 is STR consisting nine alleles with 9 to 17 repeats of AGAT motif, DYS437 is STR consisting five alleles with 13 to 17 repeats of TCTA motif, DYS4 is STR consisting 9 alleles with 6 to 14 repeats of TTTTC motif, DYS439 is STR consisting 6 alleles with 9 to 14 repeats of GATA motif, DYS448 is STR consisting 10 alleles with 17 to 24 repeats of AGAGAT motif, YGATA H4 is STR consisting 6 alleles with 8 to 13 repeats of TAGA motif [18,19], DYS437 is a tetra-nucleotide STR consisting 8 alleles with 11 to 18 repeats of TCTA motif, and DYS439 is a tetra-nucleotide STR consisting 9 alleles with 8 to 16 repeats of AGAT motif (Butler et al., 2002; Gusmao et al., 2006).

Allelic frequencies involving 17 Y-STR loci have been determined with such a necessity in a representative group of Iraq population in order to make comparisons with other populations. Seventeen (17) Y-STRs have been analyzed for diversity in 200 healthy unrelated male individuals. Observed allelic or genotype frequencies of the 17 Y-STR loci have been given in Tables 2 to 5. In this study, six alleles (20 to 25) for DYS635, three alleles (14 to 16) for DYS437, five alleles (18 to 22) for DYS448, five alleles (13 to 17) for DYS456, seven alleles (14 to 20) for DYS458, five alleles (10 to 14) for YGATA H4, three alleles (12 to 14) for DYS389I, six alleles (28 to 33) for DYS389II, five alleles (13 to 17) for DYS19, five alleles (8 to 12) for DYS391, four alleles (9 to 12) for DYS438, five alleles (21 to 25) for DYS390, six alleles (9 to 14) for DYS439, four alleles (11 to 14) for DYS392, three alleles (13 to 15) for DYS393, eight alleles (11 to 19) for DYS385a and eight alleles (13 to 20) for DYS385b were found among the whole Iraqi subjects examined.

Gene diversity values for each Y-STR loci have been given in Tables 2 to 5. Allele 10 of DYS391 or allele 13 of DYS393. It is likely that Iraqi males who belong to Y-lineages with either allele 10 or allele 13 are more susceptible to develop prostate cancer, while those belonging to lineages with allele 9 and 14 of DYS439 or allele 15 of DYS385b are more resistant to the disease. As shown in Table 6, no significant differences were observed between frequency distributions of different

Table 3. Allelic frequencies of (DYS393, DYS438, DYS439, DYS635, DYS437, DYS448, DYS456 and DYS458) genetic loci observed in 100 Iraqi males patients with prostate cancer.

Alleles	DYS393		DYS438		DYS439		DYS635		DYS437		DYS448		DYS456		DYS458	
	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.
8	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
9	·	·	0.18	0.04	0.02	0.01	·	·	·	·	·	·	·	·	·	·
10	·	·	0.54	0.07	0.46	0.07	·	·	·	·	·	·	·	·	·	·
11	·	·	0.21	0.05	0.31	0.06	·	·	·	·	·	·	·	·	·	·
12	·	·	0.07	0.02	0.05	0.02	·	·	·	·	·	·	·	·	·	·
13	0.62	0.08	·		0.15	0.04	·	·	·	·	·	·	0.09	0.03	·	·
14	0.21	0.05	·		0.01	0.01	·	·	0.42	0.06	·	·	0.06	0.02	0.04	0.02
15	0.17	0.04	·	·	·	·	·	·	0.27	0.06	·	·	0.51	0.07	0.33	0.06
16	·	·	·	·	·	·	·	·	0.31	0.06	·	·	0.23	0.05	0.11	0.03
17	·	·	·	·	·	·	·	·	·	·	·	·	0.11	0.04	0.14	0.04
18	·	·	·	·	·	·	·	·	·	·	0.29	0.06	·	·	0.08	0.042
19	·	·	·	·	·	·	·	·	·	·	0.46	0.07	·	·	0.1	0.04
20	·	·	·	·	·	·	0.1	0.03	·	·	0.1	0.03	·	·	0.2	0.05
21	·	·	·	·	·	·	0.06	0.02	·	·	0.1	0.03	·	·	·	·
22	·	·	·	·	·	·	0.04	0.02	·	·	0.05	0.02	·	·	·	·
23	·	·	·	·	·	·	0.31	0.06	·	·	·	·	·	·	·	·
24	·	·	·	·	·	·	0.39	0.06	·	·	·	·	·	·	·	·
25	·	·	·	·	·	·	0.1	0.03	·	·	·	·	·	·	·	·
26	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
28	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
30	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
31	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
33	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
D		0.985		0.991		0.989		0.99		0.989		0.989		0.989		0.989

S.E., Standard error; F., allelic frequencies.

Table 4. Allelic frequencies of (YGATA H4, DYS19, DYS385a, DYS385b, DYS389I, DYS389II, DYS390, DYS391 and DYS392) genetic loci observed in 100 Iraqi males patients without prostate cancer.

	YGATA H4		DYS19		DYS385a		DYS385b		DYS389I		DYS389II		DYS390		DYS391		DYS392	
	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.
8	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.08	0.03	·	·
9	·	·	·	·	·	·	·	·	·	·	·	·	·	·	0.09	0.03	·	·
10	0.13	0.04	·	·	·	·	·	·	·	·	·	·	·	·	0.71	0.08	·	·
11	0.21	0.05	·	·	0.11	0.03	·	·	·	·	·	·	·	·	0.05	0.02	0.53	0.07
12	0.4	0.06	·	·	0.05	0.02	·	·	0.13	0.03	·	·	·	·	0.07	0.03	0.18	0.04
13	0.12	0.03	0.13	0.04	0.44	0.06	0.1	0.03	0.42	0.06	·	·	·	·	·	·	0.16	0.04
14	0.14	0.04	0.14	0.04	0.09	0.03	0.27	0.05	0.45	0.06	·	·	·	·	·	·	0.13	0.04
15	·	·	0.51	0.07	0.07	0.03	0.03	0.02	·	·	·	·	·	·	·	·	·	·
16	·	·	0.14	0.04	0.11	0.03	0.08	0.03	·	·	·	·	·	·	·	·	·	·
17	·	·	0.08	0.03	0.05	0.03	0.16	0.04	·	·	·	·	·	·	·	·	·	·
18	·	·	·	·	0.07	0.02	0.11	0.03	·	·	·	·	·	·	·	·	·	·
19	·	·	·	·	0.11	0.03	0.16	0.04	·	·	·	·	·	·	·	·	·	·
20	·	·	·	·		·	0.09	0.03	·	·	·	·	·	·	·	·	·	·
21	·	·	·	·		·	·	·	·	·	·	·	0.16	0.04	·	·	·	·
22	·	·	·	·		·	·	·	·	·	·	·	0.17	0.04	·	·	·	·
23	·	·	·	·		·	·	·	·	·	·	·	0.08	0.03	·	·	·	·
24	·	·	·	·		·	·	·	·	·	·	·	0.5	0.06	·	·	·	·

Table 4. Contd.

25	·	·	·	·	·	·	·	·	·	·	0.09	0.03	·	·	·	·		
28	·	·	·	·	·	·	·	·	·	0.07	0.03	·	·	·	·	·	·	
29	·	·	·	·	·	·	·	·	·	0.43	0.06	·	·	·	·	·	·	
30	·	·	·	·	·	·	·	·	·	0.31	0.06	·	·	·	·	·	·	
31	·	·	·	·	·	·	·	·	·	0.11	0.03	·	·	·	·	·	·	
32	·	·	·	·	·	·	·	·	·	0.05	0.02	·	·	·	·	·	·	
33	·	·	·	·	·	·	·	·	·	0.03	0.01	·	·	·	·	·	·	
D		0.989		0.97	0.99		0.991		0.99		0.991		0.99		0.988		0.989	

S.E., Standard error; F., allelic frequencies

Table 5. Allelic frequencies of (DYS393, DYS438, DYS439, DYS635, DYS437, DYS448, DYS456 and DYS458) genetic loci observed in 100 Iraqi males patients without prostate cancer.

Alleles	DYS393		DYS438		DYS439		DYS635		DYS437		DYS448		DYS456		DYS458	
	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.	F.	S.E.
8	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
9	·	·	0.22	0.05	0.01	0.01	·	·	·	·	·	·	·	·	·	·
10	·	·	0.57	0.08	0.48	0.06	·	·	·	·	·	·	·	·	·	·
11	·	·	0.17	0.04	0.32	0.05	·	·	·	·	·	·	·	·	·	·
12	·	·	0.04	0.02	0.02	0.01	·	·	·	·	·	·	·	·	·	·
13	0.6	0.08	·	·	0.15	0.04	·	·	·	·	·	·	0.09	0.03	·	·
14	0.22	0.05	·	·	0.01	0.01	·	·	0.42	0.06	·	·	0.06	0.02	0.03	0.01
15	0.18	0.04	·	·	·	·	·	·	0.27	0.05	·	·	0.51	0.06	0.37	0.06
16	·	·	·	·	·	·	·	·	0.31	0.04	·	·	0.23	0.05	0.12	0.03
17	·	·	·	·	·	·	·	·	·	·	·	·	0.11	0.03	0.1	0.03
18	·	·	·	·	·	·	·	·	·	·	0.29	0.05	·	·	0.07	0.03
19	·	·	·	·	·	·	·	·	·	·	0.46	0.06	·	·	0.09	0.03
20	·	·	·	·	·	·	0.08	0.03	·	·	0.1	0.03	·	·	0.22	0.05
21	·	·	·	·	·	·	0.07	0.03	·	·	0.1	0.03	·	·	·	·
22	·	·	·	·	·	·	0.05	0.02	·	·	0.05	0.02	·	·	·	·
23	·	·	·	·	·	·	0.32	0.05	·	·	·	·	·	·	·	·
24	·	·	·	·	·	·	0.41	0.06	·	·	·	·	·	·	·	·
25	·	·	·	·	·	·	0.07	0.03	·	·	·	·	·	·	·	·
26	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
28	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
30	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
31	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
33	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
D		0.989		0.98		0.991		0.99		0.992		0.991		0.991		0.99

S.E., Standard error; F., allelic frequencies.

alleles of DYS635, DYS437, DYS448, DYS456, DYS458, YGATA H4, DYS389I, DYS389II, DYS19, DYS438, DYS439, DYS392, DYS385a and DYS385b loci among cases and controls.

In another study, Y-lineages of prostate cancer patients and healthy control individuals were determined for four ethnic groups living in Hawaii and California. They found one lineage, belonging to the Japanese group in the study, associated with a statistically significant predisposition to develop prostate cancer (Paracchini et al., 2003).

On the other hand, males who had either allele 3 of DYS391 or allele 25 of DYS390 showed a significantly higher risk to develop prostate cancer. These findings are consistent with those reported by Ewis et al. (2002) and Paracchini et al. (2003) which support the hypothesis that males from different Y-chromosomal origins are different concerning their susceptibility or resistance to develop prostate cancer.

In another study conducted by the current study group on Iranian population regarding comparison of Y-haplotype

Table 6. Comparison between patients and controls based on its number and frequency.

DYS loci	Allele	Patient		Control	
		No.	Freq.	No.	Freq.
YGATA H4	10	15	0.15	13	0.13
	11	23	0.23	21	0.21
	12	41	0.41	40	0.4
	13	10	0.1	12	0.12
	14	11	0.11	14	0.14
DYS19	13	28	0.28	13	0.13
	14	11	0.11	14	0.14
	15	45	0.45	51	0.51
	16	11	0.11	14	0.14
	17	5	0.05	8	0.08
DYS385a	11	13	0.13	11	0.11
	12	3	0.03	5	0.05
	13	47	0.47	44	0.44
	14	9	0.09	9	0.09
	15	4	0.04	7	0.07
	16	9	0.09	11	0.11
	17	5	0.05	5	0.05
	19	10	0.1	7	0.07
DYS385b	13	7	0.07	10	0.1
	14	31	0.31	27	0.27
	15	2	0.02	3	0.03
	16	10	0.1	8	0.08
	17	15	0.15	16	0.16
	18	10	0.1	11	0.11
	19	20	0.2	16	0.16
	20	5	0.05	9	0.09
DYS389I	12	15	0.15	13	0.13
	13	39	0.39	42	0.42
	14	46	0.46	45	0.45
DYS389II	28	10	0.1	7	0.07
	29	41	0.41	43	0.43
	30	28	0.28	31	0.31
	31	13	0.13	11	0.11
	32	2	0.02	5	0.05
	33	6	0.06	3	0.03
DYS390	21	12	0.12	16	0.16
	22	21	0.21	17	0.17
	23	6	0.06	8	0.08
	24	48	0.48	50	0.5
	25	23	0.23	9	0.09
DYS635	20	10	0.1	8	0.08
	21	6	0.06	7	0.07
	22	4	0.04	5	0.05
	23	31	0.31	32	0.32

Table 6. Contd.

	24	39	0.39	41	0.41
	25	10	0.1	7	0.07
DYS391	8	9	0.09	8	0.08
	9	6	0.06	9	0.09
	10	66	0.66	71	0.71
	11	13	0.13	5	0.05
	12	6	0.06	7	0.07
DYS392	11	58	0.58	53	0.53
	12	16	0.16	18	0.18
	13	16	0.16	16	0.16
	14	10	0.1	13	0.13
DYS393	13	62	0.62	60	0.6
	14	21	0.21	22	0.22
	15	17	0.17	18	0.18
DYS438	9	18	0.18	22	0.22
	10	54	0.54	57	0.57
	11	21	0.21	17	0.17
	12	7	0.07	4	0.04
DYS439	9	2	0.02	1	0.01
	10	46	0.46	48	0.48
	11	31	0.31	32	0.32
	12	5	0.05	2	0.02
	13	15	0.15	15	0.15
	14	1	0.01	1	0.01
DYS437	14	42	0.42	42	0.42
	15	27	0.27	27	0.27
	16	31	0.31	31	0.31
DYS448	18	29	0.29	29	0.29
	19	46	0.46	46	0.46
	20	10	0.1	10	0.1
	21	10	0.1	10	0.1
	22	5	0.05	5	0.05
DYS456	13	9	0.09	9	0.09
	14	6	0.06	6	0.06
	15	51	0.51	51	0.51
	16	23	0.23	23	0.23
	17	11	0.11	11	0.11
DYS458	14	4	0.04	3	0.03
	15	33	0.33	37	0.37
	16	11	0.11	12	0.12
	17	14	0.14	10	0.1
	18	8	0.08	7	0.07
	19	10	0.1	9	0.09
	20	20	0.2	22	0.22

lineages of prostate cancer patients and healthy control individuals comprising DYS388, DYS435, DYS437 and DYS439 loci, it was revealed that some haplotypes had higher frequency among Iranian patients than controls (unpublished data).

In a study done by Kim et al. (2007) on Korean populations of prostate cancer patients and healthy controls using Y-chromosomal binary loci, no significant difference was observed in distribution of Y-haplogroup frequencies among Korean case and control groups. Ewis et al. (2002) compared allele frequency distribution of DYS19 in Japanese prostate cancer patients and healthy controls. Based on their findings, males with allele C (194 bp) of DYS19 were more susceptible to develop prostate cancer.

Conclusion

There are some DYS lineages among Iraqi populations with significantly different allelic frequencies between prostate cancer and healthy control people, indicating that belonging to these lineages would potentially increase the level of susceptibility or resistance to prostate cancer. It is likely that Iraqi males who belong to Y-lineages with either allele 10 of DYS391 or allele 13 of DYS393 are more susceptible to develop prostate cancer.

Declaration of interest

The authors have declared that no competing interest exists.

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Full Length Research Paper

Morphological and RAPD-marker characterization of *Melia volkensii* (Gürke) *in vitro* plants regenerated via direct and indirect somatic embryogenesis

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Somaclonal variation induced *in vitro* during tissue culture can be a problem in clonal micropropagation of elite plants. This study investigated the extent of morphological and genetic similarity or dissimilarity between *Melia volkensii in vitro* plants (somatic seedlings) obtained via somatic embryogenesis and normal seedlings. Comparisons were made between *in vitro* plants regenerated directly from cotyledon explants, indirectly from zygotic embryos and normal seedlings of the same parent trees. Regeneration was achieved using half MS medium supplemented with 0.05 mg/l thidiazuron. Shoots were elongated in half MS with 0.1 mg/l BAP plus 0.01 mg/l IAA then rooted in half MS with 0.1 mg/l IBA and 0.1 mg/l NAA. Six morphometric and five meristic characters were used for the morphological characterization. PCR-RAPD markers were used for assessment of genetic similarity or distance. Multivariate analysis using principal coordinates, cluster analysis, analysis of similarities (Anosim) and similarity percentages analysis (SIMPER) revealed significant dissimilarities ($p< 0.0001$) in morphometric and meristic characters between the *in vitro* plants and normal seedlings. However, significant similarity ($p<0.01$) was observed in the RAPD-genic characters of the *in vitro* plants and normal seedlings. Out of six morphometric traits, taproot length, internode length and shoot height were the most important sources of dissimilarity, cumulatively accounting for 72.37% of overall morphometric dissimilarity. Number of lateral roots was the single most important source of meristic dissimilarity, with 77.02% contribution. Plants regenerated directly from cotyledons were more similar to the normal seedlings in morphological and RAPD-marker characters than those regenerated indirectly from zygotic embryos. This study paves the way for identification of trait-specific RAPD markers for further characterization through sequence-characterized amplified regions (SCARs).

Key words: Morphometric, meristic, PCR-RAPD, *Melia volkensii*, tissue culture, somaclonal variation.

INTRODUCTION

Melia volkensii Gürke (Meliaceae) is a drought-tolerant, fast-growing, hardwood multipurpose tree species endemic to the arid and semi-arid lands of Ethiopia, Kenya, Somalia and Tanzania (Orwa et al., 2009). The species is highly

valued for its suitability for dry land agroforestry and as a source of prized mahogany timber, termite resistant poles, animal fodder, bee forage, mulch, analgesics and botanical pesticides (Kokwaro, 1993; Stewart and Blomley, 1994; Shaalan et al., 2005; Orwa et al., 2009). *M. volkensii* outperforms other tree species found in arid and semi arid parts of Kenya, and has a higher profitability than *Eucalyptus camaldulensis* and *Grevillea robusta* in these environments (Wekesa et al., 2012). It has vast potential for arid afforestation and commercial tree farming in the region but this is hampered by difficulties in propagation via seed and vegetative means. The amenability of the species to tissue culture, first demonstrated by Indieka et al. (2007), can be exploited for mass propagation. However, tissue culture processes often induce somaclonal variations which can be problematic and require detailed characterization before the technique can be adopted for mass propagation of any plant species.

The term somaclonal variation refers to all the stable genetic, epigenetic and phenotypic variations exhibited by micropropagated plants (Larkin and Scowcroft, 1981). Some of the factors responsible for somaclonal variation include plant growth regulators, physiological and biochemical stresses imposed by the *in vitro* culture conditions, type of explant and mode of regeneration (Neelakanda and Wang, 2012). Tissue culture-induced somaclonal variation can produce off-types which are undesirable when the expected outcome is mass production of uniform genotypes (Bairu and Kane, 2011). However, it can also be a source of useful novel variations (Larkin and Scowcroft, 1981; Tang, 2005).

Somaclonal variations of genetic nature may arise from chromosomal alterations, DNA sequence changes, transposition or amplification (Neelakanda and Wang, 2012). Epigenetic variations are due to changes in gene expression arising from DNA methylation, histone modifications and small RNA-mediated regulation (Miguel and Marum, 2011; Neelakanda and Wang, 2012). DNA sequence changes can be detected by a variety of molecular markers such as random amplified polymorphic DNA (RAPD) and microsatellites or simple sequence repeats (SSR) (Jin et al., 2008), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Kour et al., 2009) and amplified fragment length polymorphism (AFLP) (Li et al., 2007).

Morphological variation is common in tissue cultured plants (Bairu et al., 2011). The extent of morphological similarity or dissimilarity between tissue cultured plantlets (somatic seedlings) and normal seedlings can be characterized using morphometric and meristic traits. Morphometrics is the quantification of variation in body form or morphology of a plant or animal using measurable characteristics (Bookstein, 1991). On the other hand, meristics is the quantification of variation in body form using counts of organs and other parts of the body (Lawing et al., 2008). Morphometric traits tend to show continuous variation and are easily influenced by the environment. Conversely, meristic traits are usually fixed early in development and are less influenced by the environment.

Recent applications of multivariate analysis on morphometric and meristic data in the study of morphological variation in plants include Valenzuela et al. (2011) and Plazas et al. (2014). Similar approaches have been used for other tissue cultured plants such as tomato (Pratta et al., 2000), soybean (Radhakrishnan and Kumari, 2008) banana (Sheidai et al., 2008) and poplar tree (Gamburg and Voinikov, 2013).

Random amplified polymorphic DNA (RAPD) (Williams et al., 1990) is a simple and quick DNA fingerprinting technique that uses a single decamer primer of arbitrary nucleotide sequence and so does not require prior knowledge about the genomic DNA being amplified. It is ideal for species with scanty elucidation of their genomic DNA, such as *M. volkensii*. The polymorphism observed in the RAPD markers is due to nucleotide sequence variations formed in the random primer binding regions of template DNA as a result of nucleotide insertion, deletion or substitution (McGregor et al., 2000). Plant species for which RAPD markers have been applied to study tissue culture-induced somaclonal variation or stability include *Cinnamomum* spp. (Soulange et al., 2007), *Mucuna* spp. (Sathyanarayana et al.. 2008), olive (Peyvandi et al., 2009), banana (Sheidai et al., 2010; Abdellatif et al., 2012).

At present, there are only two reports on the use of genetic markers to study genetic variability in natural populations of *M. volkensii* (Runo et al., 2004; Hanaoka et. al., 2012). Runo et al. (2004) used RAPD markers to compare the population genetic structure of *M. volkensii* populations in eastern and coastal regions of Kenya. Hanaoka et al. (2012) used microsatellite markers to study genetic variation in three natural populations in Kenya.

To date there are no reports on the characterization of the morphological or genetic variation in tissue cultured *M. volkensii* plants. The main objective of this study was to determine, for the first time, the extent of similarity or dissimilarity in morphological and RAPD-marker characteristics between Thidiazuron-regenerated *in vitro* plants and normal seedlings of the same parent trees. *In vitro* plants regenerated via direct and indirect somatic embryogenesis were assessed for morphological and RAPD-marker proximity to the normal seedling.

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Abbreviations: SCARs, Sequence-characterized amplified regions; **RAPD,** random amplified polymorphic DNA; **SSR,** simple sequence repeats; **PCR-RFLP,** polymerase chain reaction-restriction fragment length polymorphism; **AFLP,** amplified fragment length polymorphism; **PCOORDA,** principal coordinates analysis; **Anosim,** analysis of similarity; **SIMPER,** similarity percentage analysis.

Table 1. Morphometric and meristic characters used.

Character/unit	Description	Acronym
Morphometric measurements		
1. Shoot Height/mm	From shoot apex to base of the stem	SH
2. Internode length /mm	Mean length of first three internodes in lower half of the shoot	IL
3. Leaf Rachis Length /mm	Mean length of leaf rachis of three lowermost compound leaves	LR
4. Leaf mid-width/mm	Mean mid-leaf width of three lowermost compound leaves.	LD
5. Leaflet Length/mm	Mean length of leaflets of three lowermost leaves	LL
6. Length of Taproot /mm	From tip of taproot to base of stem	LTR
Meristic counts		
Character	**Description**	**Acronym**
1. Number of leaf nodes per shoot	Total number of leaf nodes present on the shoot	NL
2. Number of leaflets per rachis	Mean number of leaflets on each rachis, for three lowermost leaves	NLT
3. Number of lobes per leaflet	Mean number of leaf lobes or pinnules on leaflets of three lowermost leaves	NLB
4. Number of axillary bud sprouts	Total number of sprouted buds or lateral branches on the shoot	NAB
5. Number of lateral Roots	Total number of lateral roots arising from main root	NLR

MATERIALS AND METHODS

Plant materials

Seeds used as sources of explants for regeneration of *in vitro* plants and for germination into normal seedlings were obtained from the same cluster of five *M. volkensii* trees growing on a farm situated in Mavuria provenance in Mbeere, Embu county, eastern Kenya. The geo-reference coordinate of the seed collection site is 0° 46.379'S, 37° 39.308'E.

The two groups of *in vitro* plants used in the study were regenerated using the method described by Mulanda et al. (2014). One group was regenerated indirectly from calluses of mature zygotic embryos and the other directly from cotyledons. Induction medium was half Murashige and Skoog (1962) medium (MS) supplemented by 0.05 mg/l Thidiazuron (TDZ). Shoots were elongated in half MS with 0.1 mg/l 6-benzylaminopurine (BAP) and 0.01 mg/l indole-3-acetic acid (IAA), and rooted in half MS with 0.1 mg/l indole-3-butyric acid (IBA) plus 0.1 mg/l 1-naphthaleneacetic acid (NAA). Rooted plantlets with close phenotypic resemblance to normal seedlings were acclimatised and hardened for two weeks in the tissue culture lab in sterile vermiculite irrigated with half basal MS salts. They were then transferred to soil in pots, watered once with half basal MS and subsequently with sterile water. Hardening was attained through gradual and incremental opening of lids of culture bottles. The plants were maintained at 28±2°C, cool fluorescent daylight of 60 µmol photons m2 s-1 and 16 h photoperiod until they reached the 8 to 11 leaf stage.

Normal seedlings were obtained by germinating seeds under the same conditions. Initial germination of seeds was done in a substrate of sterile vermiculite and water. Prior to sowing, the seeds were scarified by nipping of the micropylar ends and slitting of the testa. This was followed by a 48 h pre-sowing soak in water at ambient temperature, with a change of the water after 24 h to get rid of phenolics. Two-leaf stage seedlings showing good vigour were transferred to soil and irrigated with sterile water till the 8 to 11 leaf stage. A total of 65 plants were used for the morphological characterisation, split as follows: 17 cotyledon regenerants, 18 zygotic embryo regenerants and 30 normal seedlings. A total of 130 plants were used for DNA extraction and RAPD analysis, split as follows: 20 Zygotic embryo non-clones, 20 cotyledon non-clones, 30 zygotic embryo clones, 30 cotyledon clones, and 30 normal seedlings. The disparity in number of plants used in morphological and RAPD characterization was caused by the smaller number of regenerants that rooted successfully and attained the 8 to 11 leaf stage set as prerequisite for the morphological study. Neither the rooting nor developmental stage constraints are applied to the RAPD study.

Morphometric and meristic characters used

Eleven morphological characters were used to characterize the extent of similarity or distance among and between the *in vitro* plants (regenerants) and normal seedlings. These characters were divided into two groups consisting of six morphometric measurements and five meristic counts (Table 1). Morphometric variables were measured to the nearest mm using a ruler. In order to minimize the effects of size-related differences and variations caused by the allometric nature of plant growth, the developmental stage of the somatic and normal seedlings was standardised at the 8 to 11 leaf-stage.

PCR-RAPD marker characterization

DNA extraction

The terminal portions of shoots of *in vitro* plants and normal seedlings were cut and 0.3 g fresh mass of the same used for DNA extraction. The tips were ground to a fine white powder in liquid nitrogen and 2 ml of extraction buffer added. Genomic DNA (g-DNA) was then extracted from the homogenates using the CTAB method of Doyle and Doyle (1987), with slight modifications. Extracted DNA was stored at -20°C in 40 µl of pH 8 Tris-EDTA (TE)

Table 2. Details of the decamer primers used for RAPD analysis.

Primer code name	Nucleotide sequence and GC content (%)		Related species and studies where same primer sequence was used
	5'	3'	
MEL-1	GGC ACT GAG G	(70)	Neem (*Azadirachta indica* A. Juss), meliaceae, da Silva et al. (2013)
MEL-2	GTA GAC CCG T	(60)	
MEL-3	CAA TCG CCG T	(60)	
MEL-4	CCT TGA CGC A	(60)	*Lansium domesticum* Corr., meliaceae, Song et al. (2000)
MEL-5	TGC CGA GCT G	(70)	
MEL-6	GGC ATG ACC T	(60)	Genus *Xylocarpus: X. granatum* Koen., *X. moluccensis* Lamk. and *X. mekongensis* Pierre, meliaceae, Pawar et al. (2013).
MEL-7	TGG CGC AGT G	(70)	
MEL-8	GGC TAT CCG A	(60)	Arbitrary sequence (present authors)

buffer until required for gel runs or PCR. Presence of genomic DNA was confirmed by electrophoresis.

Genomic DNA confirmation and quantification

RNA was removed from the samples by adding 2 µl of RNAase cocktail (consisting of 500 units/ml of RNAase A and 20,000 Units/ml of RNAase T1 [Ambion®]) to 20 µl of nucleic acid-TE buffer mixture. Samples were incubated for 30 min at 37°C followed by 15 min at 65°C for inactivation of the RNAase. RNA removal was confirmed by electrophoresis. RNA-free DNA was quantified and assessed for purity using a Thermoscientific Nanodrop 2000 Spectrophotometer. Readings of DNA concentration in ng/µl, together with the A_{260}/ A_{280} and A_{260}/A_{230} absorbance ratios were obtained for assessment of purity as suggested by ThermoScientific (2014).

Screening of RAPD primers

Eight random decamer primers sourced from Inqaba Biotech Industries (Pty) Ltd., South Africa, were screened for ability to amplify 10 samples of *M. volkensii* genomic DNA (5 g-DNA samples from wild type seedlings and 5 from tissue-cultured plants). The oligonucleotide sequences of seven of the primers (Mel-1 to Mel-7) were selected from lists of primers used in other RAPD-marker studies done on tree species belonging to three genera of the meliaceae family; *Azadirachta, Lansium* and *Xylocarpus* (Song et al., 2000; daSilva et al., 2013; Pawar et al., 2013). The eighth primer (Mel-8) is an arbitrary sequence chosen by the present authors. This approach was used because the only previous report of RAPD-markers in *M. volkensii* (Runo et al., 2004) did not reveal sequences of the primers used. Table 2 gives details of the primer sequences used in the present study and the related species where they were used.

PCR-RAPD amplification

The composition of the PCR reaction mixture and thermal cycler conditions were optimized by varying the type of Taq polymerase (Dream Taq® or Taq polymerase® from ThermoScientific, and MyTaq® from Bioline), concentration of Taq polymerase (0.75, 1.0, 1.25 and 2.5 units per 25 µl reaction), strength of PCR buffer (x1 or x1.5), concentration of primer (25 or 50 pmoles per 25µl reaction), annealing temperature (34, 35, 36, 37 and 38°C) and extension time (1 or 2 min). The optimum amplification reaction mixture per 25 µl reaction consisted of 5 µl of 5x Bioline MyTaq® reaction buffer, 0.5 µl Bioline MyTaq® DNA polymerase, 5 µl of 10 mM primer, 3.0 µl of g-DNA in TE buffer (after diluting the g-DNA-TE mixtures to half-strength using equal volume of PCR water) and 11.5 µl of nuclease-free PCR water (Sigma-Aldrich). Final concentrations of components in each 25 µl reaction were x1 MyTaq® buffer containing 1 mM dNTPs and 3 mM $MgCl_2$, 50 pmoles of primer, 2.5 units of MyTaq® DNA polymerase and 20 to 25 ng of gDNA. The polymerase chain reaction was carried out in a PTC-100 thermal cycler (Programmable Thermal Controller- MJ Research Inc., USA). Optimum PCR was attained with 5 min initial denaturation at 95°C, followed by 35 cycles consisting of 30 s denaturation at 94°C, 45 s annealing at 35°C, 60 s extension at 72°C and a single final extension of 5 min at 72°C.

Electrophoresis and visualization of gels

All electrophoresis runs were done for 45 min at 80 V (5.5 volts/cm) in a Pharmacia Biotech GNA horizontal tank. The g-DNA samples were run in 0.8% (m/v) agarose gel whilst PCR products were run in 1.0% (m/v) agarose gel, both in x1 TAE buffer. Gels contained 3 µl of 10 mM ethidium bromide as visualization stain. Wells were loaded with 7 µl DNA sample or PCR product and 3 µl of x6 bromophenol blue loading dye. A 1 kb ladder (Bioline® Hyperladder), with size markers from 200 to 10,037 base pairs, was used as molecular marker. The gels were visualized at 302 nm using a 2 UV Transilluminator and photographed using the integrated Multi Doc-it Digital Imaging System.

Data analysis

Morphometric and meristic data analysis

Morphological and meristic data were analyzed using the PAleontological STatistics (PAST) software version 2.17c (Hammer et al., 2001). Morphometric data was subjected to logarithmic transformation to minimise size-related differences, as suggested by Palmer and Strobeck (2003). No such transformation was performed for meristic data as meristic characters are usually independent of size of the organism, and tend to be fixed in the early stages of growth. The data were subjected to multivariate analysis as detailed below.

RAPD data scoring and analysis

Only clearly resolved bands were scored. The RAPD bands/markers were scored for each lane using 1 for presence and

Table 3. Variation in morphological characters (Mean ± standard error).

Population	SH	IL	LR	LL	LD	LTP
Morphometric measurements (mm)						
ZE regenerants	43.83±4.88	4.68±0.76	15.28±1.16	30.44±1.92	21.18±1.66	8.33±8.33
Cotyledon regenerants	51.00±3.58	5.54±0.56	14.04±1.85	30.65±1.96	22.60±1.15	27.41±8.17
Wild type	187.81±8.07	28.06±2.68	31.78±1.43	72.50±2.28	63.79±2.55	131.2±3.59
Meristic counts						
Population	**NL**	**NLT**	**NLB**	**NAB**	**NLR**	
ZE regenerants	10.06 ± 0.30	6.20 ± 0.33	7.01 ± 0.24	0.06 ± 0.06	0.00 ± 0.00	
Cotyledon regenerants	9.88 ± 0.29	5.77 ± 0.27	6.47 ± 0.21	0.06 ± 0.06	2.88 ± 1.04	
Wild type	8.41 ± 0.13	5.14 ± 0.18	7.64 ± 0.18	0.21 ± 0.12	24.11 ± 1.34	

SH= Shoot length; IL= Internode length; LR= Leaf rachis length; LD= Mid-of-leaf width; LL= Leaflet length; LTP= Length of taproot. NL = Number of leaf nodes per shoot; NLT= Number of leaflets per compound leaf; NLB= Number of lobes/pinnules per leaf; NAB= Number of axillary bud sprouts per shoot; NLR= Number of lateral roots per root.

0 for absence of a band. The resultant binary matrix of 1 and 0 scores was used for analysis. The matrix data was analyzed using the Popgene Population Genetic Analysis software Version 1.32 (Yeh et al., 2000). Six summary genic variation statistics were obtained; observed number of alleles (N_a), effective number of alleles (N_e), Nei's (1973) gene diversity index (H), Shannon's information index (I), number of polymorphic loci (N_p) and percentage of polymorphic loci (P_p). Also generated was a matrix of Nei's (1978) unbiased genetic similarity and genetic distance values between the groups.

Multivariate analyses

The log-transformed morphometric data, raw meristic data and the Popgene-generated genic variation values were subjected to the following multivariate analyses in PAST version 2.17c: Principal Coordinates analysis (PCOORDA) and Cluster analysis, one-way Analysis of Similarity (Anosim) and Similarity Percentage analysis (SIMPER) (Hammer et al., 2001). Principal axes values and Eigenvalues obtained were used to plot PCOORDA scatterplots and 95% confidence ellipses using the Gower measure of distance. This measure of distance is applicable to mixed type data (continuous and ordinal) (Hammer et al., 2001). Clustering was performed using the paired-group algorithm and Gower measure of distance, with 1000 bootstrap replications. Anosim and SIMPER were done using Bray-Curtis measure of similarity, which is the default measure. Pair-wise percentages of dissimilarity between groups were computed using Anosim, with 10,000 permutations. The Anosim test statistic R measures the overall dissimilarity between groups. R values closer to zero indicate no dissimilarity (significant similarity), those closer to 1 indicate significant dissimilarity between the groups. Individual and cumulative contributions of the characters to the overall average percentage dissimilarity were obtained using SIMPER.

RESULTS AND DISCUSSION

Morphological variation

Significant morphological variation was observed between the regenerants and wild seedlings in morphometric and meristic traits. In the morphometric characters, the regenerants differed significantly from wild type seedlings in all the six traits used (Table 3). Significant stunting of shoots occurred in both zygotic embryo (indirect) regenerants and cotyledon (direct) regenerants. As shown in Table 3, mean shoot heights of the two types of regenerants (43.83 and 51.00 mm) were one-quarter and one-third, respectively, of the mean shoot height of wild type seedlings (187.81 mm). The respective mean internode lengths (4.68 and 5.54 mm) of the regenerants were one-sixth and one-third of the internode length of wild type seedlings (28.06 mm) (Table 3). The leaves of the two types of regenerants also had considerably shorter rachis (14.04 and 15.28 mm), leaf lengths (30.44 and 30.65 mm) and mid-leaf widths (21.18 and 22.60 mm), in comparison to the respective mean rachis length, leaf length and mid-leaf width (31.48, 72.50 and 63.79 mm) of the normal seedlings. However, the greatest difference in morphometric traits was seen in the mean length of taproots, with taproot length of wild seedlings (131.20 mm) being on average nearly 16 times longer than that of zygotic embryo regenerants (8.33 mm) and nearly 5 times longer than that of cotyledon regenerants (27.41 mm). These results are not unusual as similar stunting of shoots regenerated using TDZ has been reported in grapes (Gray and Benton, 1991) and in tree species such as *Cassia* (Parveen and Shahzad, 2010), *Myrica rubra* (Asghari et al., 2013) and *Calophyllum inophyllum* (Thengane et al., 2006). TDZ use has also been associated with failure of regenerated shoots to root well and general inhibition of rooting (Gray and Benton, 1991; Lu, 1993; Ranyaphia et al., 2011).

As for meristic characters, there was less morphological difference between the regenerants and wild type seedlings (Table 3). Out of the five meristic traits used, the mean numbers of four of them namely number of leaves per shoot, number of leaflets per rachis, number of leaf pinnules per leaflet and number of axillary bud

Table 4. Concentration and purity ratios of extracted DNA

Group	DNA concentration (ng/µl)	A260/A280 ratio	A260/A230 ratio
Cotyledon regenerants	915.40±86.59	1.81±0.04	1.33±0.28
Zygotic embryo regenerants	379.70±104.04	1.60±0.02	0.68±0.03
Wild type seedlings	1528.32±148.60	1.83±0.03	1.69±0.08

Values are means ± standard error.

Table 5. Differences in genic variation revealed by candidate primers with 10 samples of g-DNA.

Primer	Bands/loci resolution	Bands/loci per lane	N_a	N_e	H	I	N_p	P_p
Mel-1	Low	4.89±1.05	1.30±0.47	1.17±0.34	0.09±0.18	0.15±0.25	9	30.00
Mel-2	Low	4.40±0.69	1.13±0.35	1.05±0.18	0.03±0.11	0.05±0.16	4	13.33
Mel-3	Low	5.56±0.88	1.17±0.38	1.10±0.28	0.06±0.15	0.09±0.21	5	16.67
Mel-4	High	5.00±0.00	1.10±0.31	1.05±0.19	0.03±0.09	0.04±0.14	3	10.00
Mel-5	High	7.11±2.52	1.23±0.43	1.16±0.35	0.09±0.18	0.13±0.25	7	23.33
Mel-6	High	5.00±0.00	1.00±0.00	1.00±0.00	0.00±0.00	0.00±0.00	0	0.00
Mel-7	Low	3.29±0.49	1.03±0.18	1.01±0.06	0.01±0.04	0.01±0.07	1	3.33
Mel-8	High	5.00±0.00	1.00±0.00	1.00±0.00	0.00±0.00	0.00±0.00	0	0.00

Number of distinct RAPD band types/markers = 30. N_a= observed number of alleles; N_e=Effective number of alleles; H=Nei's gene diversity index; I=Shannon's information index; N_p= Number of polymorphic alleles; P_p= Percentage polymorphism (Means ± standard deviation).

sprouts were generally similar between regenerants and wild seedlings. Only one meristic trait, mean number of lateral roots per plant, was different with the zygotic embryo regenerants having complete absence of lateral roots, cotyledon regenerants having a mean of 2.88 lateral roots per plant and the wild seedlings having a mean of 27.41 lateral roots (Table 3). In general, there was significant inhibition of lateral root formation in the regenerants. Overall, inhibition of rooting was greater in *in vitro* plants regenerated indirectly from zygotic embryos than those regenerated directly from cotyledon explants, suggesting a possible role of the mode of regeneration in determination of rooting response. The poor root meristics of both types of regenerants can also be attributed to the reported inability of plants regenerated by TDZ to root well (Gray and Benton, 1991; Lu, 1993; Ranyaphia et al., 2011).

RAPD-produced genic variation characters

Both the yield and purity levels of the extracted DNA were good (Table 4). The A_{260}/A_{280} ratios for the g-DNA of cotyledon regenerants (1.81) and that of wild type seedlings (1.83) were close to the ideal ratio of 1.80 expected for pure DNA as suggested by ThermoScientific (2014). However, the A_{260}/A_{280} ratio for the zygotic embryo regenerants (1.60) was lower than ideal indicating some contamination with proteins, whose peak absorbance is usually at 280 nm. A_{260}/A_{230} ratios obtained for the three groups of plants ranged from 0.68 to 1.69 and were much lower than the ideal ratio of 2.0 to 2.2 suggested by ThermoScientific (2014). Low A_{260}/A_{230} ratios are often encountered in plants due to a considerable carrying-over of carbohydrates during extraction. This indicates that the ratio of CTAB to sodium chloride used in this study may have failed to precipitate all the carbohydrates.

All the eight primers tested amplified *M. volkensii* g-DNA and generated a grand total of 371 monomorphic and polymorphic RAPD bands/markers giving a mean tally of 46.38 bands per primer for the 10 g-DNA sample tested. Overall amplification frequency for the total of 80 PCR-RAPD primer-screening reactions carried out was 69 out of 80 (86.25%) (Table 5). The mean number of bands/loci per lane was 5.03±1.39. Mel-5 primer, with an oligonucleotide sequence of 5' TGC CGA GCT G 3', produced the highest number of highly resolved bands (9) in 50% of the amplified samples (Figure 1), the largest mean number of bands/loci per lane (7.11±2.52) and the second highest overall percentage of polymorphic loci (23.33) (Table 5). Consequently, Mel-5 was selected as the best primer among those tested for amplification of g-DNA of the regenerants and wild seedlings. Altogether, the eight screened primers produced a total of 30 polymorphic RAPD bands/markers from the 69 amplified DNA samples. This is consistent with the findings of Runo et al. (2004), where eight random primers generated 38 scorable polymorphic bands from 90 PCR-RAPD reactions using DNA obtained from *in situ* populations of *M. volkensii.* However, a detailed comparison of primer performance between that study and the present one was not possible as Runo et al. (2004) did not reveal

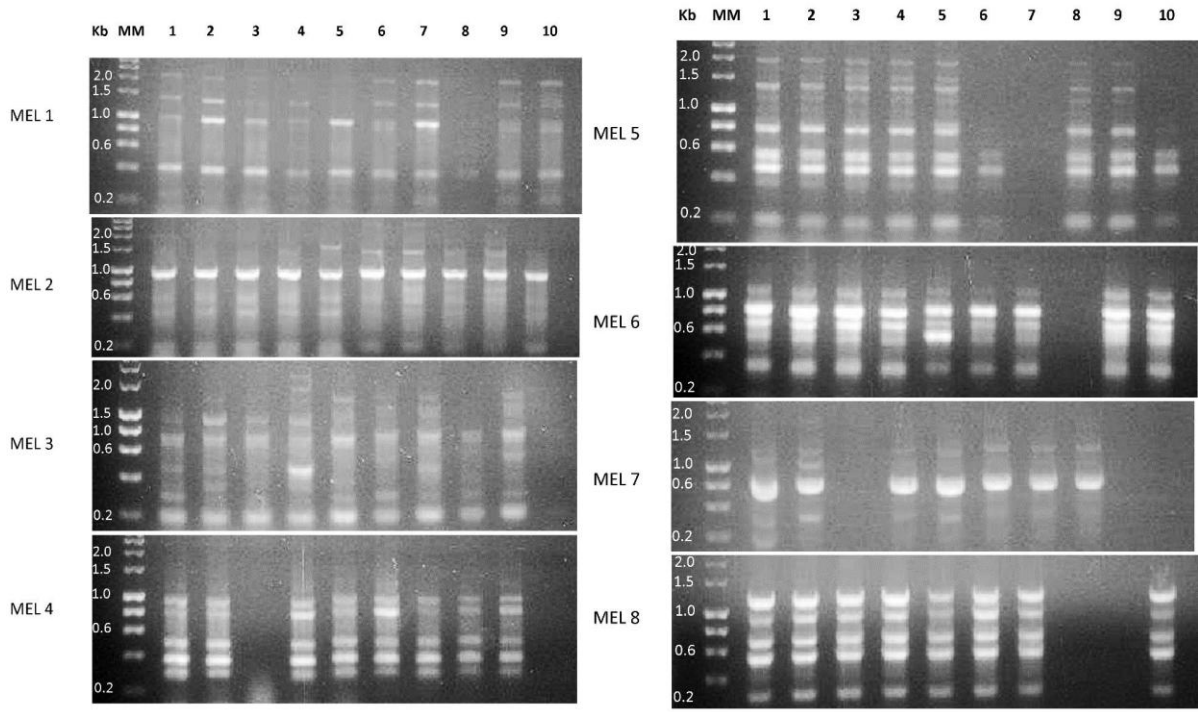

Figure 1. PCR-RAPD profiles of the eight candidate primers showing amplified and non-amplified lanes in 10 samples of g-DNA.

oligonucleotide sequences of primers used.

Amplification frequency for the selected primer (Mel-5) was 107 out a combined total of 130 PCR reactions (82.31%) for the regenerants and wild seedlings. From the 107 reactions, this primer generated a grand total of 759 monomorphic and polymorphic RAPD bands, giving a mean of 7.09 bands per lane. There were 19 distinct band sizes or loci ranging from 200 to 2000 base pairs with 17 of them being polymorphic. Two out of six genic characters namely observed number of alleles (N_a) and effective number of alleles (N_e), showed little difference between the regenerants and wild seedlings (Table 6). However, the range of Nei's gene diversity index (H) and Shannon's information index (I) was considerably greater in the regenerants (H= 0.09 – 0.18; I= 0.13 – 0.28) than the values obtained for wild type seedlings (H= 0.02; I= 0.03). In addtition, these two measures of diversity (H and I) were generally higher in the non-clone regenerants than in the cloned ones. Nei's gene diversity index was 9 to 9.5 and 4.5 to 6.5 times greater, respectively, in non-cloned and cloned regenerants than in wild type seedlings (Table 6). Shannon's information index was 9.33 and 4.36 times greater, respectively, in non-cloned and cloned regenerants than in wild type seedlings. The number of polymorphic loci and percentage of polymorphic loci each were 9 to 11 and 4 to 5 times greater, respect-tively, in non-cloned and cloned regenerants than in wild type seedlings (Table 6).

Nei's genetic variation index (H) describes variation within and between populations whereas Shannon's index (I) mainly describes variation within populations (Hennink and Zeven, 1991). A value of 0 occurs when populations are genetically identical whereas the value of 1 is for different species. The closeness to 0 obtained for both indices in the present study suggests a generally low level of genetic diversity within and between the regenerants and wild seedlings. However, since the regenerants gave higher values of Nei's gene diversity and Shannon's information indices than wild type seedlings, this indicates that the two regeneration systems used in the present study produced some genetic variations. The occurrence of such genetic variation during tissue culture is reported in other plant species (Larkin and Scowcroft, 1981; Neelakanda and Wang, 2012)

The values of Nei's gene diversity (H) index obtained for the regenerants in this study (0.09 to 0.19) compare favourably with those obtained by Runo et al. (2004) (c.a. 0.06 to 0.12) for widely dispersed natural populations of *M. volkensii* in Kenya. This indicates that genetic variability attributable to the regeneration systems used in this study may not significantly deviate from that which is found within and between natural populations of the species. The very low value of Nei's gene diversity index (0.02) seen in the wild type seedlings (Table 6) could be attributed to the fact that seeds used in raising the wild type seedlings for the RAPD analysis were from a cluster of parent trees standing on the same farm, which are probably closely related.

Table 6. RAPD-based genic characteristics obtained with selected primer for regenerants and wild seedlings

Population	Amplified/total DNA samples	N_a	N_e	H	I	N_p	P_p (%)
ZE non-clones	19/20	1.58±0.51	1.30±0.37	0.18±0.19	0.28±0.28	11	57.89
Cotyledon non-clones	17/20	1.47±0.51	1.33±0.41	0.19±0.22	0.27±0.31	9	47.37
ZE clones	22/30	1.26±0.45	1.25±0.42	0.13±0.22	0.18±0.31	5	26.32
Cotyledon clones	21/30	1.21±0.42	1.16±0.33	0.09±0.18	0.13±0.26	4	21.05
Wild type	28/30	1.05±0.23	1.03±0.12	0.02±0.07	0.03±0.12	1	5.26
Overall	107/130	1.90±0.03	1.45±0.04	0.26±0.02	0.39±0.03	30	31.58

Number of distinct RAPD bands/loci=19. ZE= Zygotic embryo. N_a= observed number of alleles; N_e=Effective number of alleles; H=Nei's gene diversity index; I=Shannon's information index; N_p= Number of polymorphic alleles; P_p= Percentage polymorphism (Means ± standard deviation).

Principal coordinates (PCOORDA) analysis

Principal Coordinate Analysis (PCOORDA) for the morphological characters revealed a clear separation of the regenerants from the wild type (Figure 2a, b). The 95% confidence ellipses showed a greater difference between the regenerants and wild type seedlings in morphometric traits than in meristic traits. This was supported by the variance contributions of the principal axes and corresponding Eigenvalues, which explained 81.87% of the observed differences in morphometric traits but only explained 48.42% of the differences seen in meristic traits. For both types of morphological characters, the largest separation from the wild type was seen in regenerants obtained from zygotic embryos via callus-mediated (indirect) somatic embryogenesis. Plants regenerated from cotyledons via direct somatic embryogenesis were morphologically closer to the wild type as shown by larger overlap of ellipses. The PCOORDA plot for RAPD-based genic variation characters showed little or no genetic differentiation between the regenerants and the wild plants. There were very large overlaps of the 95% confidence ellipses of the regenerants and wild type seedlings (Figure 2c). Lack of clear separation of the regenerants and normal seedlings based on RAPD-genic variation characters could suggest that the morphological differences observed in this study may be epigenetic or physiological as suggested by Miguel and Marum (2011) and Bairu and Kane (2011).

Cluster analysis

Cluster analysis also showed a clear separation of the regenerants from wild type seedlings based on morphological characters but no significant separation in genic characters (Figures 3a, b). When clustered based on morphometric traits, there were two distinct clusters. Wild type seedlings formed the first distinct cluster with 84% bootstrap reliability. The second cluster grouped together the regenerants with a lower bootstrap value of 48%. There was a high level of within-cluster overlap among and between the two types of regenerants, showing high similarity and little differentiation of the regenerants in morphometric traits. Clustering using meristic traits also produced two significant clades, a smaller clade with 58% bootstrap reliability consisting of only three wild type seedlings and a much larger clade with 53% bootstrap reliability, in which most of the wild type seedlings and the two types of regenerants were clustered (Figure 3b). Within-cluster overlap was greater in meristic than morphometric traits for the wild type and the two types of regenerants. This indicates that wild type seedlings and regenerants had a higher level of within-group similarity in meristic traits than in morphometric characters.

Clustering based on genic variation characters revealed complete lack of genetic distance in 66 out of the total of 96 (68.75%) plants tested and presence of some genetic differentiation in the remainder (31.25%) (Figure 4). The no-distance clade consisted of 94.7% of all the wild type DNA samples tested, 71.4% of the entire DNA from cotyledon clones, 63.6% of the entire DNA from zygotic embryo clones, 52.6% of DNA from cotyledon non-clones and 42.9% of DNA from zygotic embryo non-clones. Consequently, based on this clustering, the largest genetic differentiation between regenerants and wild type seedlings occurred in zygotic embryo non-clones at 57.1% followed by cotyledon non-clones at 47.4%. Zygotic embryo clones had 36.4% genetic differentiation from wild type whilst the value for cotyledon clones was 28.4%, making cotyledon clones to be the closest in genetic similarity to the wild type seedlings.

The use of cluster analysis to complement either principal coordinate or principal component analyses of morphological or genetic traits in plants is well documented (Pratta et al., 2000; Song et al., 2000; Sheidai et al., 2008, 2010; Valenzuela et al., 2011; Abdellatif et al., 2012; Gamburg and Voinikov, 2013; Plazas et al., 2014). In the present study cluster analysis validated the PCOORDA plots. Regenerants were morphologically separate from, but genetically similar to, the normal seedlings. This seems to support the view that

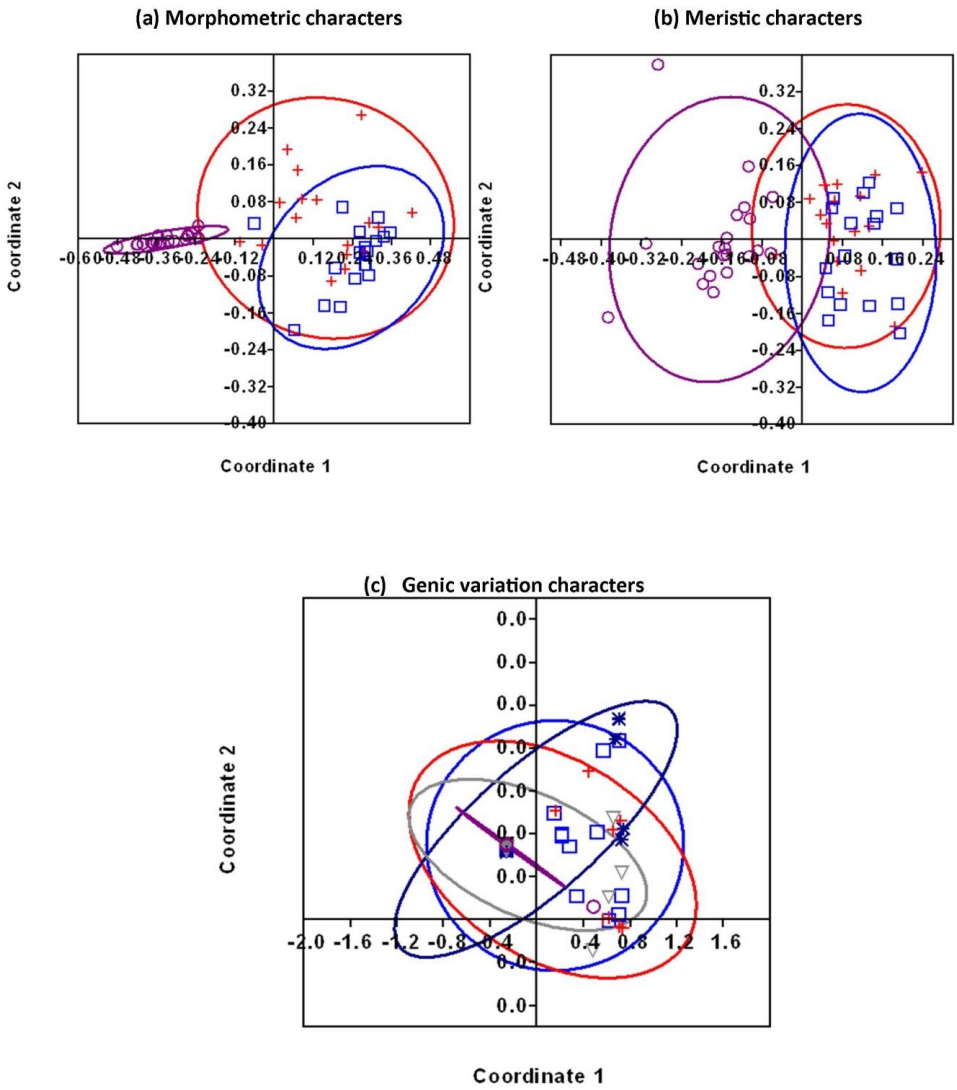

Figure 2. PCOORDA scatter plots and 95% confidence ellipses. **(a) and (b)** Zygotic embryo regenerants (□), cotyledon regenerants (+), wild type (○). **(c).** zygotic embryo non-clones (□), zygotic embryo clones (•), cotyledon non-clones (Δ), wild type (○).

morphological differences observed in this study may have arisen from epigenetic or physiological factors as described by Miguel and Marum (2011) and Bairu and Kane (2011).

Analysis of similarity (Anosim) and similarity percentages (SIMPER)

Anosim results further validated the PCOORDA and cluster analysis results. A similar complementary role of the Anosim test to principal coordinate analysis is reported by Abouzied et al. (2013). Morphometric and meristic traits gave Anosim R-values of 0.778 ($p<0.0001$) and 0.688 ($p<0.0001$), respectively, indicating significant morphological dissimilarity (or distance) between the regenerants and wild seedlings (Table 7). However, RAPD-based genic variation characters gave an Anosim R-value of 0.047, showing no significant genetic dissimilarity (or significant similarity) between the regenerants and wild seedlings. The SIMPER analysis carried out for a pair-wise comparison of dissimilarities between groups revealed that morphological dissimilarities were generally greater between wild seedlings and regenerants than between the two types of regenerants themselves (Table 8).

Morphological dissimilarities were greatest between wild type seedlings and zygotic embryo (indirect) regenerants, at 27.10% for morphometric and 41.47% for meristic traits respectively. Dissimilarity percentages for genic variation characters averaged around 20% between wild type seedlings and non-clone regenerants on

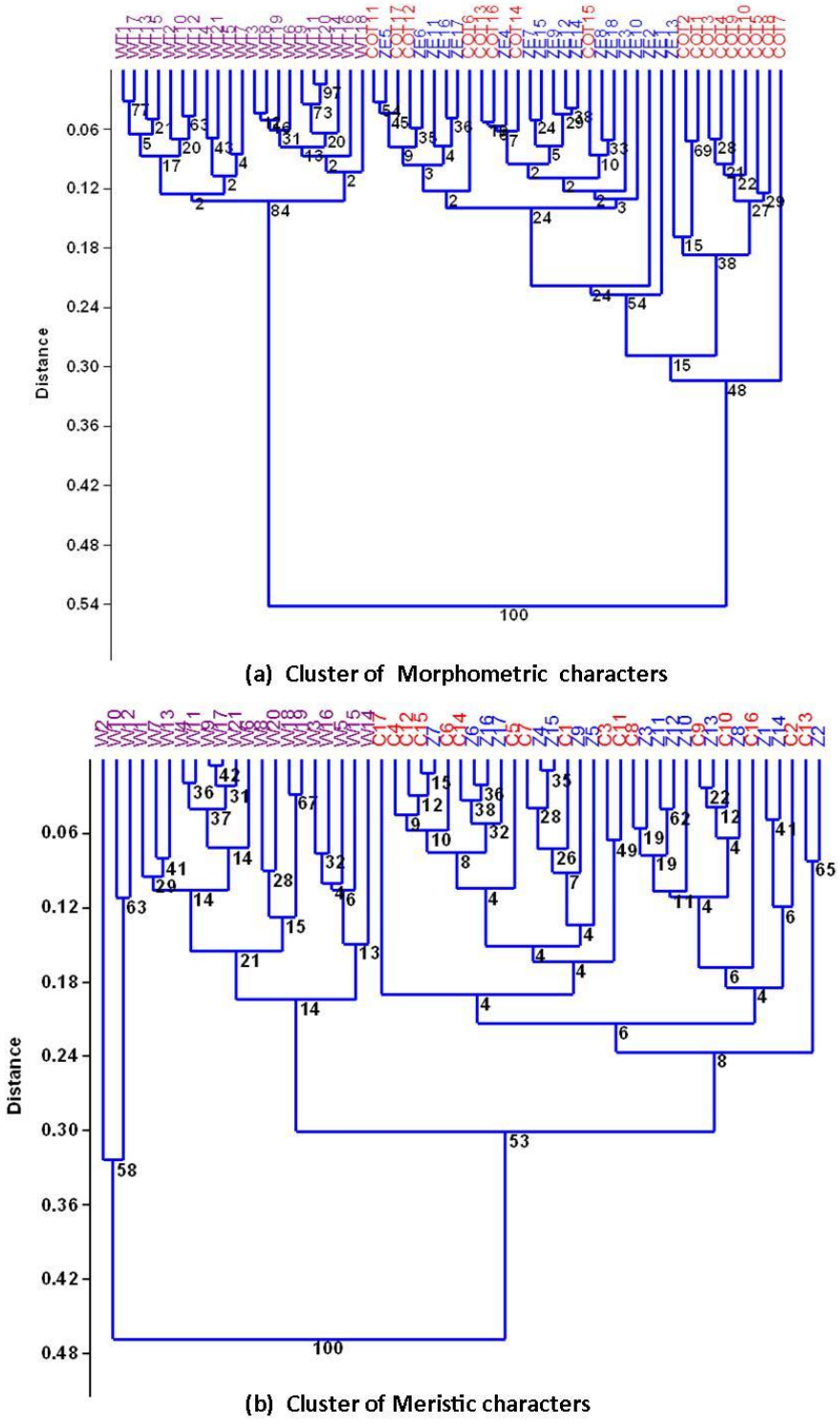

Figure 3. Paired-group clusters for (a) morphological (b) and meristic characters. Node values are bootstrap percentages for 1000 replications. W/WT= wild type seedlings; Cot/ C= cotyledon regenerants; ZE/Z= zygotic embryo regenerants.

one hand and 9.82 to 15.61% between wild type seedlings and cloned regenerants on the other hand. This indicates a high level of genetic similarity (84.39 to 90.18%) between the cloned *in vitro* plants and normal seedlings of the parent trees (Table 8) and less similarity between none-clones and normal seedlings. Out of the

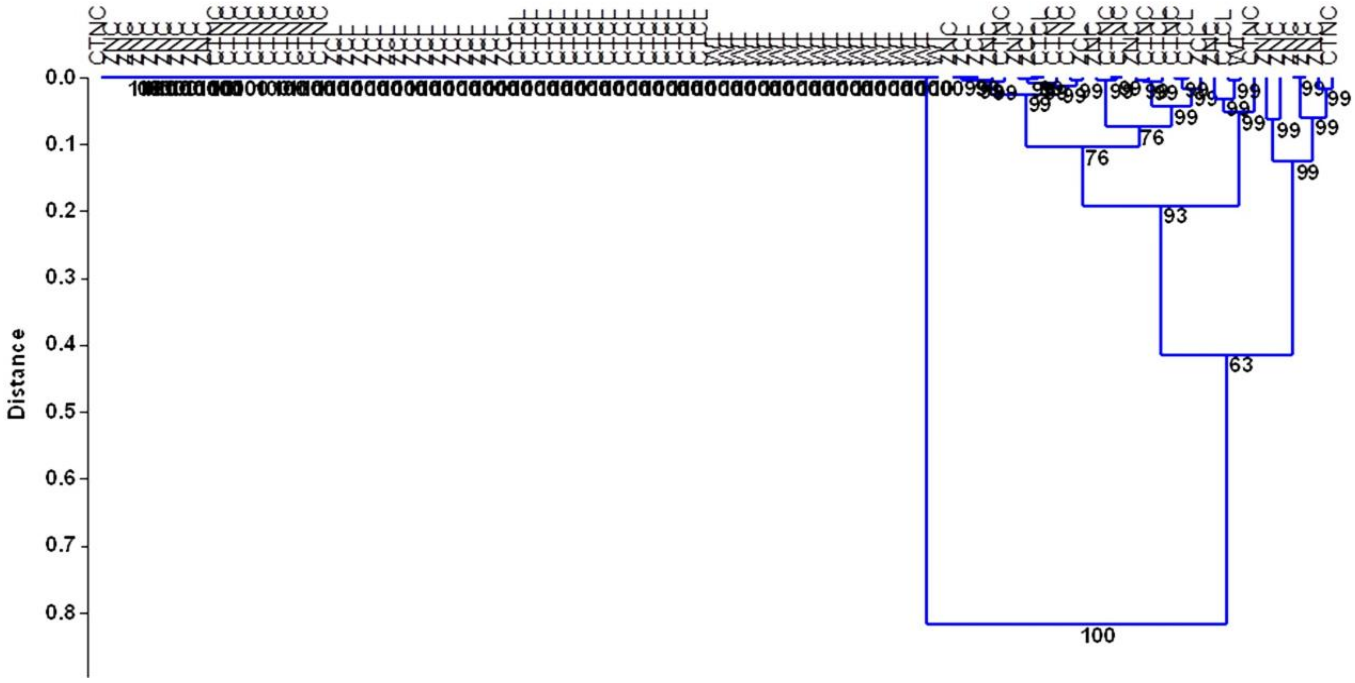

Figure 4. Paired-group clusters for genic characters. Node values are bootstrap percentages for 1000 replications. (CTNC= cotyledon non-clones, CTCL=cotyledon clones, ZNC= zygotic embryo non-clones, ZCL= zygotic embryo clones, WT= wild type seedlings).

Table 7. One-way Anosim results for morphological and RAPD-marker data.

Parameter	Morphometric characters	Meristic characters	RAPD-marker genic variation characters
Mean Rank within	365.8	397.8	2147
Mean Rank between	964.5	908.9	2253
R value	0.778	0.688	0.047
p(same)	0.000099	0.000099	0.0077
Inference	Significant dissimilarity between groups	Significant dissimilarity between groups	Significant similarity between groups

overall average morphometric dissimilarity observed, length of taproot alone accounted for 41.52% (Table 9). This was followed by internode length (17.25%) and shoot height (13.60%). These three traits accounted for 72.37% of the overall dissimilarity in morphometric characters. As for the meristic traits, the mean number of lateral roots was the single most important source of dissimilarity, accounting for 77% of the dissimilarity, followed by the mean number of leaves and leaflets at 7.82 and 7.17%, respectively. These three traits accounted for 91.99% of the overall dissimilarity in meristic characters. Observed number of alleles was the single most important source of dissimilarity among the genic variation characters, accounting for 37.65% of the dissimilarity. This was followed by effective number of alleles and Shannon's information index at 26.22 and 21.45%, respectively. These three variables accounted for 85.32% of the overall dissimilarity in genic characters. On its own, Nei's gene diversity index accounted for only 14.68% of the overall average dissimilarity. This shows that based on Nei's gene diversity index alone, the genetic similarity between the regenerants and wild type seedlings can be estimated at 85.32%.

Conclusions and recommendations

In vitro plants of *M. volkensii* regenerated via both direct and indirect TDZ-induced somatic embryogenesis differed significantly from seedlings of the same mother trees in morphological characters. However, RAPD markers did not reveal significant genetic difference. The regeneration systems used in the study can therefore be considered to be capable of maintaining high genetic fidelity and proximity

Table 8. Pair-wise SIMPER dissimilarity percentages for morphological and genic characters

Morphometric characters (%)			
	Cotyledon regenerants	**ZE regenerants**	**Wild type**
Cotyledon regenerants	0		
ZE regenerants	12.92	0	
Wild type	20.92	27.1	0

Meristic characters (%)			
	Cotyledon regenerants	**ZE regenerants**	**Wild type**
Cotyledon regenerants	0		
ZE regenerants	13.44	0	
Wild type	36.15	41.47	0

Genic variation characters (%)					
	Cotyledon non-clones	**Z.E non-clones**	**Cotyledon clones**	**Z.E clones**	**Wild type**
Cotyledon non-clones	0				
Z.E non-clones	21.17	0			
Cotyledon clones	19.90	21.13	0		
Z.E clones	20.50	21.59	15.61	0	
Wild type	19.01	20.80	12.41	9.82	0

Table 9. SIMPER ranking of individual and cumulative contributions of characters to overall average dissimilarity between the groups.

Character	**Average dissimilarity**	**Contribution %**	**Cumulative contribution %**
Morphometric characters			
1 Length of tap root	8.64	41.52	41.49
2 Internode length	3.59	17.25	58.77
3 Shoot height	2.83	13.60	72.37
4 Mid-of-leaf diameter	2.15	10.33	82.70
5 Leaf length	1.82	8.75	91.45
6 Leaf rachis length	1.78	8.55	100
7 Overall average dissimilarity	20.81	-	-
Meristic characters			
Character	**Average dissimilarity**	**Contribution %**	**Cumulative contribution %**
1 Number of lateral roots	24.26	77.02	77.02
2 Mean number of leaf nodes per shoot	2.46	7.81	84.83
3 Mean number of leaflets per leaf	2.26	7.17	92.00
4 Mean number of pinnules/lobes on leaflets	2.12	6.73	98.73
5 Number of axillary bud sprouts on shoot	0.40	1.27	100
6 Overall average dissimilarity	31.50	-	-
Genic variation characters			
Character	**Average dissimilarity**	**Contribution %**	**Cumulative contribution %**
1 Observed number of alleles	6.85	37.66	37.66
2 Effective number of alleles	4.77	26.22	63.88
3 Shannon's information index	3.90	21.44	85.32
4 Nei's gene diversity	2.67	14.68	100
5 Overall average dissimilarity	18.19	-	-

to the parent trees. These are the first such findings for *M. volkensii.* Observed morphological differences may be due to epigenetic or physiological causes. Further molecular characterisation using other markers such as microsatellite (SSR) and amplified fragment length polymorphism (AFLP) may be required for confirmation of the apparent genetic similarity between the regenerants and normal seedlings. Use of methylation-sensitive PCR is recommended for detection of epigenetic changes. We also recommend the identification of RAPD markers that may be uniquely associated with particular morphological traits and the subsequent development of sequence-characterised amplified regions (SCARs).

Conflict of interest

The authors declare no conflict of interest.

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Full Length Research Paper

Effect of temperature, salinity, light and time of dehiscence on seed germination and seedling morphology of *Calotropis procera* from urban habitats

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Calotropis procera **(giant milkweed) is a hardy xerophytic plant, which is distributed globally in many countries and has important economic and ecological functions. The present study aimed at estimating the effect of temperature, salinity and time of fruit dehiscence on the seed germination and seedling morphology of giant milkweed in urban habitats. Seeds were collected early (in June) and late (in December) from pre-dehiscent (EPRD, LPRD) and post-dehiscent (EPOD, LPOD) fruits. Mature seeds were 100% viable, while premature seeds did not germinate. The highest germination and mean time to germination was attained in full dark followed by dark/light, while no germination occurred at complete light. A significant difference between seeds from pre- and post-dehiscent fruits was assessed, while no significant difference between early and late seeds. Maximum germination was at 25 and 30°C, while germination was inhibited at 35°C, which demonstrates that temperature is one of the critical factors for giant milkweed seed germination. Moreover, salinity more than 2000 mg l^{-1} NaCl inhibited seed germination. It is likely that high temperature, direct light conditions and high salinity are the limiting factors for the establishment of giant milkweed seeds. The germination of *C. procera* seeds was significantly affected when germinated with *Trigonella foenum-vulgare* with the reduction of germination from 100 to 34%, while the later plant was not affected. *C. procera* is considered as an important medicinal plant; therefore, our results provide useful information for its management under different environmental conditions.**

Key words: Giant milkweed, seed morphology, fruit dehiscence, germination time and viability.

INTRODUCTION

Germination requirements and dormancy characteristics of species are often assumed to be adaptations to the particular habitat, where the species occur (Angevine and Chabot, 1979; Meyer et al., 1990). Germination at the right

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Abbreviations: EPRD, Early pre-dehiscent; **EPOD,** early post-dehiscent; **LPRD,** late pre-dehiscent; **LPOD,** late post-dehiscent.

time and in the right place is important to determine the probability of a seedling surviving to maturity (Thompson, 1973). Seed germination and early seedling growth are critical stages for the establishment of plant populations under saline conditions (Perez et al., 1998; Khan and Gulzar, 2003). Salinity and temperature have differential effects on seed germination (Khan and Gulzar, 2003, Al-Khateeb, 2006). In temperate regions, many plant species have evolved physiological mechanisms in the seeds to detect the appropriate time for germination (Washitani and Masuda, 1990; Baskin and Baskin, 1998). Eco-physiological studies of seed germination have suggested that temperature, its changes, and its fluctuation can be the most reliable environmental signals to indicate the appropriate timing for germination (Baskin and Baskin, 1998).

Calotropis procera (Asclepiadaceae) is a perennial shrub that primarily reproduces via seeds. It is native to tropical and subtropical Africa and Asia, and common in the Middle East (Parsons and Cuthbertson, 2001; Lottermoser, 2011). *C. procera* (also known as Sodom apple, giant milkweed, cabbage tree and rubber tree) is a xerophytic shrub or small tree. It grows as a wasteland weed and flowers and fruits profusely every year from March to May. This plant can tolerate adverse climatic conditions and poor soils. It has an evergreen behavior under field conditions, from young plant to the reproductive stage (Frosi et al., 2012). The fruit dehisces along the ventral suture at maturity while still green dispersing on an average of 230 ± 19 silky haired seeds (Lottermoser, 2011). *C. procera* is well known to attract/support about 80 animal species ranging from casual visitors to those fully dependent on the plant for completion of their life cycle (Sharma and Amritphale, 2008; Boutraa, 2010). It had been widely used in traditional medicine due to its pharmacological active compounds found in all plant parts; barks, roots, leaves and especially its milky latex which exudates from damaged and broken leaves and stems (Magalhaes et al., 2010). A wide range of chemical compounds have been isolated from giant milkweed, including cardiac glycosides, flavonoids, phenolic compounds, terpenoides (Mueen Ahmed et al., 2005). Because of these active chemical compounds, *C. procera* has been used for treatment of a number of diseases, such as ulcers, tumors, leprosy, piles and diseases of the spleen, liver and abdomen. Furthermore, *C. procera* is optionally used for animal feed during dry periods in the semi-arid regions (Boutraa, 2010).

Rarity of endangered plant species can be strongly related to human threats and environmental limiting factors, and understanding of these factors is a necessity to protect the vegetation of these plant species. Further climate change with high temperature associated with dry conditions might be an important factor in reducing the success of regeneration of a number of plant species. Another factor that can greatly affect plant diversity is the herbivore grazing (Wei et al., 2009). Few studies were carried out on the seed germination of *C. procera* (Sen et al., 1968; Sharma and Amritphale, 2008). Some studies were conducted under controlled (Boutraa, 2010) or field conditions from ecological and ecophysiological perspectives (Khan et al., 2007; Oliveira et al., 2009; Tezara et al., 2011). To provide basic information for its conservation and restoration in natural and urban habitats, we studied the influence of environmental factors on seed germination patterns. The germination responses of seeds as well as seedling growth were determined over a wide range of temperatures, light intensity and salinity gradients. In addition, the effect of the time of fruit dehiscence on the seed germination of *C. procera* was also tested. A bioassay test was conducted to investigate the effect of giant milkweed seed germination on the germination of economic plants seeds.

MATERIALS AND METHODS

Seed collection

Mature and premature seeds, from post-dehiscent and pre-dehiscent fruits, respectively, of *C. procera* were collected during June (early) and December (late) 2012, from several plants belonging to different populations which are naturally growing in the urban habitats in Helwan region, Cairo, Egypt. Seeds were collected from pre-dehiscent (manually opened) and post-dehiscent (naturally opened) fruits. Fruits and seeds characteristics such as length, width and weight as well as number of seeds per fruit were measured. After removing the seed silky hairs, which had little effect on seed germination in the preliminary studies (Sharma and Amritphale, 2008), the seeds were allowed to desiccate in paper bags for three days at room temperature, washed twice with distilled water, and then stored at room temperature in dry and dark conditions after sterilization with 0.5% sodium hypochlorite solution for 1 min.

Viability test, light, temperature and salinity experiment

The viability of premature and mature seed was tested by 2,3,5-triphenyltetrazolium chloride (TZ) method (ISTA, 1985). To determine the optimal light requirements for seed germination, 15 seeds (three replicates) were placed in sterilized 9 cm Petri dishes containing two layers of filter papers moistened with distilled water, and incubated at 25°C at full light, equal light: dark and full dark. Light intensity was 1985 lux (PPFD = 26.8 µmol m^{-2} s^{-1}). The effect of temperature on seed germination was tested in sterilized Petri dishes, where a set of 15 seeds (five replicates) was subjected to the following temperatures: 15, 20, 25, 30 and 35°C in dark (optimum conditions). For each treatment, seeds were gathered from early pre-dehiscent (EPRD), early post-dehiscent (EPOD), late pre-dehiscent (LPRD) and late post-dehiscent (LPOD) fruits. For studying the effect of salinity on seed germination, another set of 15 seeds (five replicates) was placed in Petri dishes containing two layers of filter paper at different concentrations of NaCl (500, 1000, 2000, 3000 and 4000 mg l^{-1}) and incubated at 25°C in dark (optimum conditions). For each treatment, growth parameters (shoot and root length) were recorded at the last day of the germination experiments. The seed was considered to have germinated when the radical emerged from the seed coat. Germination was monitored until no more new seed germination was observed over seven consecutive days.

Table 1. Light requirements for germination and mean time to germination (MTG) of *Calotropis procera* seeds.

Variable (light treatment)	Germination (%)	MTG (days)
Light	0[a]	0[a]
Light / dark	82.7±2.9[b]	18.7±3.9[b]
Dark	98.7±16.7[c]	24.1±0.5[c]

Values are mean ± SD (n = 3). Different letters within a column indicate that means are significantly different among light treatments ($p < 0.05$).

Table 2. Germination and mean time to germination (MTG) of *Calotropis procera* seeds at different temperature treatments.

Temperature (°C)	EPOD	EPRD	LPOD	LPRD	F-value
Germination (%)					
20	0[a]	1.3±2.9[a]	54.7±27.6[a]	22.7±12.1[a]	14.2**
25	96.4±7.6[b]	22.7±18.0[b]	93.3±8.2[b]	50.7±23.9[bd]	27.0**
30	80.0±19.4[c]	49.3±12.9[c]	85.3±20.2[b]	61.3±15.9[bc]	4.6*
35	65.3±15.2[d]	6.7±6.7[a]	80.0±21.1[ab]	33.3±10.5[ad]	25.9**
MTG (days)					
20	0[a]	0.1±0.2[a]	6.9±3.2[a]	2.5±1.3[a]	18.0**
25	22.1±2.2[b]	2.6±2.5[a]	22.8±2.5[b]	12.1±5.7[bd]	35.8**
30	19.0±5.1[bc]	19.9±25.9[b]	20.9±5.4[b]	14.5±4.5[bc]	0.2
35	15.9±3.7[c]	0.8±0.7[a]	19.1±5.5[b]	7.3±2.9[ad]	26.4**

EPRD: Early pre-dehiscent, EPOD: early post-dehiscent, LPRD: late pre-dehiscent and LPOD: late post-dehiscent fruits. Values are mean ± SD (n = 3). Different letters within a column indicate that means are significantly different among temperature treatments. *: $P < 0.01$ and **: $P < 0.001$.

Bioassay test

A bioassay test was conducted to estimate the effect of germinating *Calotropis* seeds altogether with seeds from another economic plant (*Trigonella foenum-vulgare*) at 25°C using five replicates (10 seeds for each species). Growth parameters (shoot and root length) were recorded at the last day of the germination experiments.

Data analysis

The mean time to germination (MTG) was calculated as: MTG = $\frac{\sum(ni \times di)}{N}$, Where, ni is the number of seeds germinated at day i (di), and N is the total number of seeds germinating in the treatment (Redondo-GÓmez et al., 2008). After testing the data for normality, the differences in the germination percentage, MTG as well as growth variables among the different treatments were tested using one-way analysis of variance (ANOVA) using SPSS software (SPSS, 2006). A post-hoc test was applied when treatment effects were significant.

RESULTS

The giant milkweed's fruits were more or less rectangular in shape with a mean length of 12 cm and a mean width of 10 cm. In addition, the fruit weighted about 40 g. Moreover, each fruit could produce about 410 seeds with a mean seed length of 6.6 mm and width of 4.5 mm, while the weight of 10 seeds was about 0.1 g. Seed viability test indicated a 100% germination of *C. procera* mature seeds, while premature seeds did not germinate. Seed germination varied significantly according to their light requirements (Table 1). The highest seed germination (98.7%) and mean time to germination (MTG = 24.1 days) was obtained at dark conditions, while no germination occurred at full light treatments. The study of *C. procera* seed germination at different temperature treatments indicated significant variation between the different seeds, while mean time to germination was significant different at all temperatures, except at 30°C (Table 2). *C. procera* seeds from EPOD fruits had significant higher germination percentage (96.4%) at 25°C, while those from LPOD had no significant differences between 25 and 30°C (93.3 and 85.3%, respectively). On the other hand, germination of EPRD and LPRD seeds were significantly affected ($p < 0.05$) by temperature variation with the highest germination percentage at 30°C. Moreover, no germination occurred at 15°C for all seeds, while the highest temperature (35°C) showed notable reduction in the seed germination.

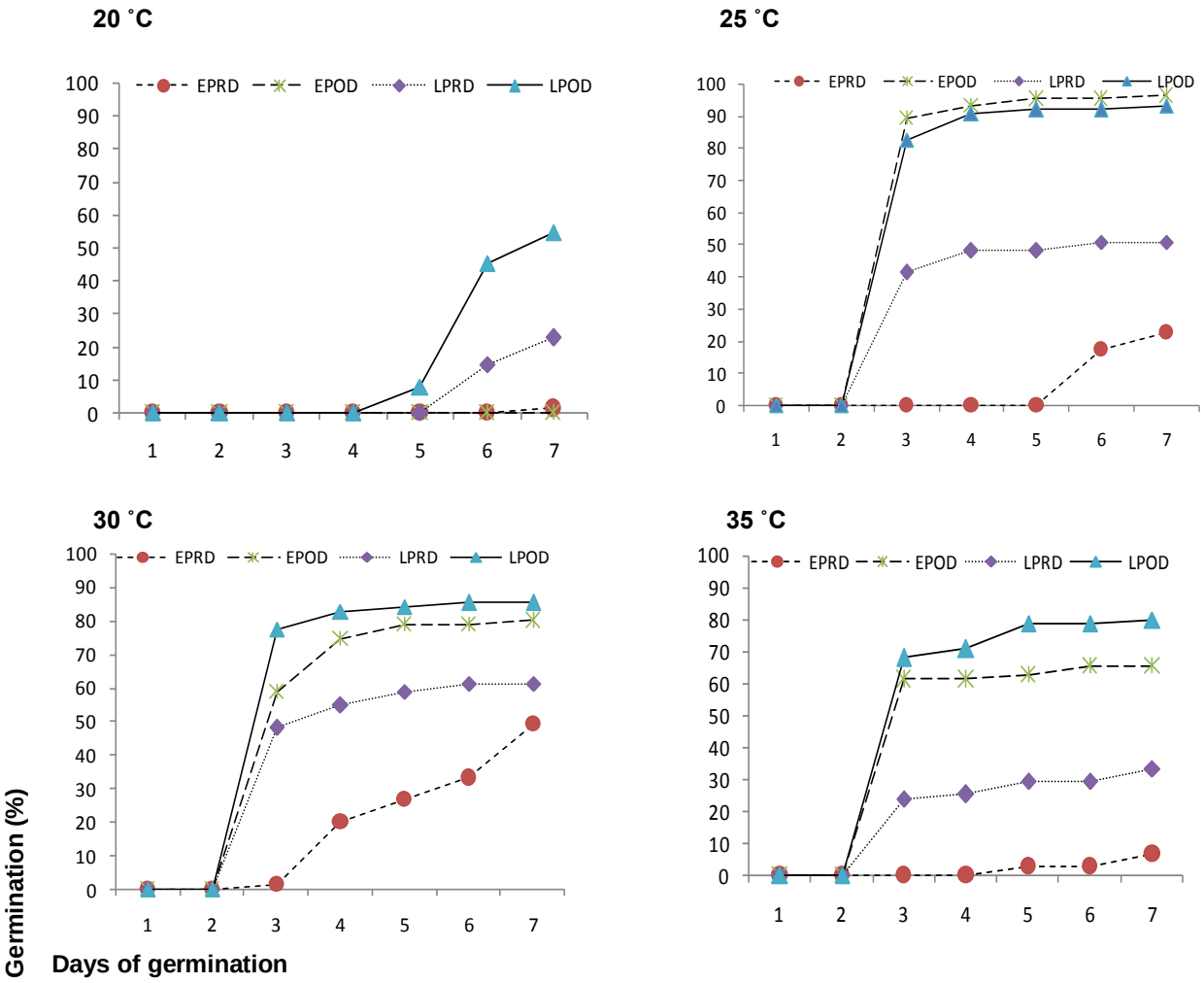

Figure 1. Cumulative mean germination percentage of *Calotropis procera* seeds collected from different fruits at different maturity stages. EPRD: Early pre-dehiscent, EPOD: early post-dehiscent, LPRD: late pre-dehiscent and LPOD: late post-dehiscent fruits.

EPOD and LPOD seeds had significantly the longest MTG at 25°C (22.1 and 22.8, respectively) and 30°C (19.0 and 20.9) with no significant difference between them. All seeds, except EPRD, germinated considerably at the third day of incubation at 25, 30 and 35°C (Figure 1). At these temperature treatments, the seed germination (%) was in the order: EPOD > LPOD > LPRD > EPRD. Temperature variation significantly affected the shoot and root lengths of the germinated seedlings (Figure 2). The maximum growth of *C. procera* seedlings after seven days was found at 30°C, with no significant difference between shoot length of EPRD, LPRD and LPOD, but shoots of EPOD were significantly shorter.

In addition, the longest root was obtained at 30°C, where LPOD had significantly the longest. Decreasing temperature significantly decreased the shoot and root lengths of *C. procera* seedlings. Percentages of seed germination at different concentrations of NaCl after seven days of incubation were not significantly different between NaCl concentrations of 500, 1000 and 2000 mg l^{-1} and Control (Table 3). Conversely, seed germination at NaCl concentrations of 3000 and 4000 mg l^{-1} was significantly lower than at the lower concentrations. It is apparent that at 500 mg l^{-1} and 1000 mg l^{-1} NaCl concentrations, the fastest germination was obtained, MTG= 24.1 and 24.2, respectively, whereas germination at 3000 and 4000 mg l^{-1} was significantly retarded. The shoot length of *C. procera* seedlings, after seven days of incubation, is not greatly affected by salinity variations (Table 3). The shoot length at 500 mg l^{-1} NaCl was significantly lower compared to the other salinity treatments that had no significant differences between them. On the other hand, the root length of *C. procera* seedlings was significantly decreased by increasing NaCl concentrations.

The germination of *C. procera* seeds was significantly

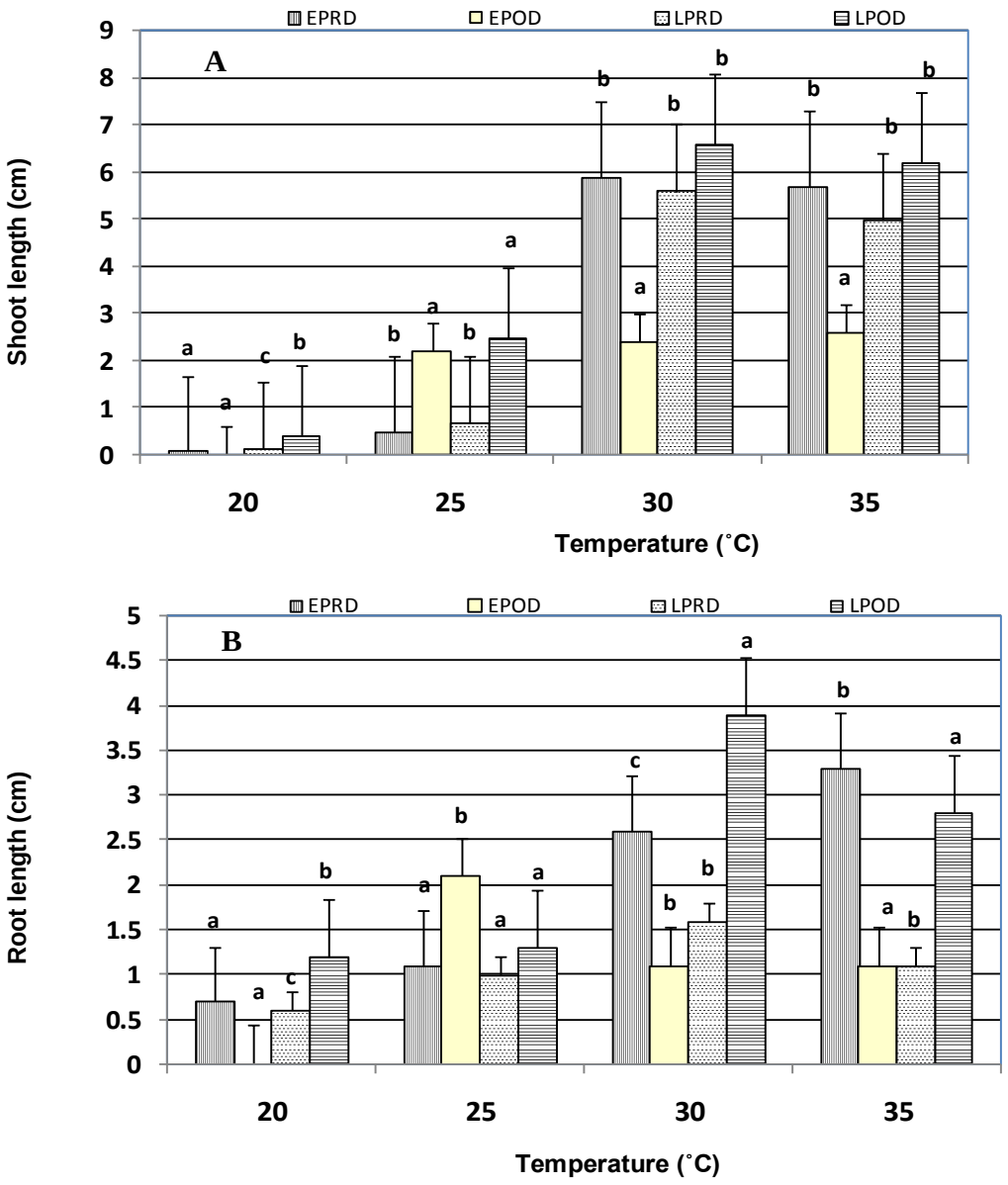

Figure 2. Shoot (A) and root (B) lengths of Calotropis procera seedling after 7 days of seed germination at different temperatures. Lines over bars represent SE. EPRD: Early pre-dehiscent, EPOD: early post-dehiscent, LPRD: late pre-dehiscent and LPOD: late post-dehiscent fruits.

affected when germinated with *T. foenum-vulgare* with the reduction of germination from 100 to 34%, while the later plant was not affected (Table 4). On the other hand, the shoot length of *Calotropis* and *Trigonella* seedlings was significantly larger in combination than if each species are alone. Moreover, the root length of both species was not significantly affected.

DISCUSSION

An increase in atmospheric temperature, particularly in tropical regions, appears to be an inevitable consequence of global climate change. This is likely to affect plant diversity and performance, owing to the relationship between temperature and seed germination, and temperature and plant vigor (Perumal et al., 2014). Seed germination of xerophytes is regulated by factors such as water, temperature, light, soil salinity and their interactions (Noe and Zedler, 2000); however, each species responds to the abiotic environment in a unique manner (Khan and Gulzar, 2003). Due to the importance of *C. procera* in medicine, pharmacology and environment; documentation of its seed germination and effects of different climatic conditions on growth performance and other physiological and biochemical aspects are encouraged

Table 3. Effect of salinity on germination, mean time to germination (MTG) and seedling growth of *Calotropis procera* seeds.

NaCl treatment (mg l^{-1})	Germination (%)	MTG (days)	Shoot length (cm)	root length (cm)
0	98.7±8.0[a]	22.7±3.2[a]	3.1±0.4[a]	2.0±0.5[a]
500	100±0.0[a]	24.1±0.5[a]	2.2±0.4[b]	1.4±0.3[b]
1000	100±0.0[a]	24.2±0.6[a]	2.7±0.3[a]	1.6±0.3[b]
2000	88±8.7[a]	21.3±2.3[a]	2.7±0.6[a]	1.3±0.3[b]
3000	73.3±23.6[b]	16.4±4.8[b]	2.8±0.3[a]	1.5±0.3[b]
4000	33.3±13.3[c]	7.7±3.3[c]	2.9±0.1[a]	1.4±0.1[b]

Values are mean ± SD (n = 3). Different letters within a column indicate that means are significantly different among salinity treatments ($p < 0.05$).

Table 4. Seed germination and growth parameters of *Calotropis procera* and *Trigonella foenum-vulgare* seedlings germinated at 25 °C.

Treatment	*Calotropis*	Mixed		*Trigonella*
		Calotropis	*Trigonella*	
Germination (%)	100[a]	34[b]	32[c]	52.5[c]
Shoot length (cm)	0.5±0.2[a]	1.3±0.5[b]	2.2±1.5[c]	6.0±0.5[d]
Root length (cm)	1.1±0.6[a]	1.9±0.5[ac]	1.8±0.4[c]	2.3±0.8[c]

Values are mean ± SD (n = 3). Different letters indicate that means are significantly different among treatments ($p < 0.05$).

(Boutraa, 2010). Our investigation showed that seeds collected only from mature fruits were viable, while premature seeds failed to germinate. The high viability of seeds gives opportunity to the plant to establish whenever it finds the proper habitats. Francis (2003) reported that seedlings of *C. procera* often grow in large numbers after rainy periods, but only a few survive the first season.

Light is an important factor in seed germination because in many seeds, phytochrome-mediated responses play a critical role in determining the time of germination and thus become a crucial part of the evolutionary strategy to impose conditional dormancy to protect seedlings from environmental extremes (Gula et al., 2013). Pronounced inhibition of germination was observed in the seeds of giant milkweed exposed to light conditions, while the highest germination percentages were obtained in dark. This means that without complete burial in the ground, the seeds of Calotrope could not germinate and establish in its habitats. This helps in explaining why Calotrope populations distribute as single fewer individuals in the field (El-Middany, 2014). It was reported that seeds of only few shrubs, including *Artemisia monosperma, Artemisia ordosica, Artemisia sphaerocephala* (Huang and Gutterman, 1999; 2000), are highly sensitive to light for germination. However, similar to several dark-germinating seeds, for example, *Amaranthus caudatus, Cucumis sativus,* and several cultivars of *Lactuca sativa*, germination of mature non-dormant seeds of *C. procera* was inhibited by intermittent infra-red light, though more frequent daily exposures were required (Sharma and Amritphale, 2008). Such retardation of germination by exposure to light has been reported to occur in the seeds of many other plants (Baskin and Baskin, 1998). In addition, the mechanism behind this light inhibition is somehow related to red/infra-red light, which is operative in many seeds or due to the accumulation of some toxic products of respiration (Thornton, 1935).

Temperature can affect the percentage and rate of germination through its effects on loss of dormancy and the germination process itself (Roberts, 1988). Reduced temperatures and critical high temperature would be expected to retard the metabolic rate to the point where pathways essential for the onset of germination would cease to operate (Verma et al., 2010; Kumar et al., 2011). The influence of temperature on germination in this study indicated that seeds of *C. procera* have a narrow range of temperature to germinate. Irrespective to the time of harvesting, temperature of 25 and 30°C seemed to be optimum for seed germination of giant milkweed seeds. According to Baskin and Baskin (1998), temperature requirements for shrubs in hot semi-deserts and deserts to achieve 60 to 100% germination range from 15 to 35°C, with temperatures of about 20 to 25°C being suitable for most species.

The germination percentage was declined by increasing or decreasing the temperature more than the optimum. It looks that seeds collected from EPOD and LPOD fruits had more germination success than the pre-dehiscent ones. Nevertheless, the present study furnishes the information regarding the relation between temperature regime and seed germination, taking only the time of harvesting into considerations while other

factors are constant. The present study indicates that giant milkweed seeds failed to germinate at 15°C and attained lower percentages at both 20 and 35°C than at 25 and 30°C. We think that the germination capacity of these seeds may be altered significantly if they are exposed to high-temperature conditions during germination. In summer, temperature is usually 35°C or higher and the soil temperature may be in many places, where the species is distributed, higher than air temperature (for example, in exposed sandy soil). It seems to be a critical period up to which the embryo can withstand the high temperature conditions and the germination capacity of the seeds may fall with longer duration before rain. It was reported that many seeds of shrubs, succulents, and forbs germinated in the arid lands following rains in autumn and early winter, when temperatures were decreased (Huang et al., 2003). It is apparent from the results of the present study that *C. procera* has a relative salt tolerance during its germination stages, but this tolerance is low under high NaCl concentrations. According to Francis (2003) and Orwa et al. (2009), giant milkweed is known to be a drought-resistant, salt-tolerant species and it is capable of surviving in a range of soil types, including alkaline and saline soils. This is obvious in the distribution of this species in the habitats close to River Nile and its adjacent desert areas, which are characterized by relative low salt content compared to salt treatments in the present study (El-Midany, 2014). Moreover, the inhibitory effects of NaCl on seed germination could be due to its direct effect on the growth of the embryo (Al-Khateeb, 2006).

In the life cycle of a plant, seeds have the highest resistance to extreme environmental stresses, whereas seedlings are most susceptible, and this is especially true for desert species (Gutterman, 1993). Therefore, successful establishment of a plant population is dependent on the adaptive aspects of seed germination and of early seedling growth (Qu et al., 2008). The shoot length of *C. procera* seedlings was not greatly affected by salinity variations while the root length was greatly affected in saline compared to the control treatment. Similar to Poljakoff-Mayber et al. (1994), the most influential effect of salt was on the root, which may delimit the germination success and establishment of the seeds in their habitats. They found that the elongation of the embryonic axis of *Kosteletzkya virginica* was strongly inhibited by high levels of NaCl in irrigation solutions. Alternatively, NaCl may also increase the osmotic potential of the media causing inhibitory effects on seed imbibitions (Poljakoff-Mayber et al., 1994; Al-Khateeb, 2006). Salt tolerance during germination and early seedling stages is critical for the establishment of plants which can grow in saline soil (Perez et al., 1998; Al-Khateeb, 2006).

The germination of *C. procera* seeds was significantly affected when germinated with *T. foenum-vulgare* with the reduction of germination from 100 to 34%, while the latter plant was not affected. *C. procera* had inhibitory effect on germination rate and seedling length of wheat, radish and canola (Abdel-Farid et al., 2013). It plays an important role in the formation of its natural habitats as it contains the allelochemical compounds that enable the plant to compete with other species (Al-Zahrani and Al-Robai, 2007).

Conclusions

Our study indicates that direct light and high salinity inhibit the germination of *C. procera* seeds. The period after seed dispersal (in summer months) seems very critical for the plant either due to absence of rain in the study area or high temperature (≥35°C) which inhibits seed germination. Temperatures between 25 and 30°C are optimum for seed germination. *C. procera* has a relative salt tolerance during the germination stages but this tolerance was reduced under high salinity. Plant roots were more sensitive to salinity than shoot system during germination. It seems that high temperature, direct light conditions and high salinity are limiting factors for the establishment of its seeds. Only fully mature seeds can germinate successfully regardless of the harvesting date.

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Full Length Research Paper

Influence of various carbohydrates on the *in vitro* micropropagation of *Nauclea diderrichii* (De Wild &T. Durand) Merrill, an endangered forest species in Togo

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The over exploitation of *Nauclea diderrichii*, for its very resistant wood against the attacks of fungus (*Coriolus versicolor*), Lyctus, termites (*Reticulitermes santonensis*) and marine borers, leads to its disappearance in Togo. This forest species produces many fruits containing numerous seeds; unfortunately their seedling is laborious in its biotope. *In vitro* micropropagation trials were carried out for a faster and massive regeneration. The effect of carbon source on the rooting and the growth of seedlings were studied in the presence of sucrose, glucose, fructose, mannitol, maltose, galactose, mannose, lactose, sorbose and sorbitol. Woody plant medium (WPM) of Lloyd and McCown has been used. This medium was modified by supply of microelements and vitamins of Murashige and Skoog and supplemented by 30 g/L of these carbohydrates. The best plants' growth and rooting were obtained with sucrose. Sucrose appears to be the most favorable sugar to ensure the *in vitro* micropropagation of *N. diderrichii*. Sorbose caused the necrosis of explants in culture. The mannose and the mannitol, did not cause the necrosis of explants, but their presence had an unfavorable role on roots initiation and slow down the plants' growth.

Key words: Sources of carbon, *Nauclea diderrichii*, *in vitro* micropropagation, Togo.

INTRODUCTION

The composition of the culture medium determines the *in vitro* growth of plants. Sugar in culture medium has been considered the sole carbon source for the growth of cells, buds, shoots, and even plantlets (Gauchan, 2012). The

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main components of most plant tissue culture media are mineral salts and sugar as carbon source and water (Gamborg and Phillips, 1995). Sugar is a very important component in medium and its addition is essential for *in vitro* growth and development of plants because photosynthesis is insufficient, due to the weak development of their leaves, the limited gas exchange and the high relative humidity (Pierik, 1987; Kozai, 1991; Mazinga et al., 2014). Organized tissues show a better growth and proliferation after the addition in medium an adequate source of carbon (Mazinga et al., 2014). Sugars enter the metabolism pathways and transformation of energy which are required for growth of cell (Gauchan, 2012). Except sucrose which is more often used in tissue culture, others carbohydrates are successfully used as a source of carbon. However, the same carbohydrate can give very controversial effect according to the species. The fructose gave good results for culture of *Morus alba* (Oka and Ohyama, 1986), *Castanea sativa* (Chauvin and Salesses, 1988) and *Malus pumila* (Welander et al., 1989), but it gave bad results in *Malus* Jork (Moncousin et al., 1992), *Syringa chinensis* (Welander et al., 1989) and *Prunus cerasus* (Borkowska and Szczerba, 1991). As for the glucose, it showed successful results in *Alnus* (Welander et al., 1989), *Potentilla fruticosa* and *Ficus lyrata* (Wainwright and Scrace, 1989). The effect of carbohydrates on the micropropagation can differ according to the species (Baskaran and Jayabalan, 2005). We therefore performed *in vitro* tests to study the effects of diverse sources of carbon in *N. diderrichii* during the present survey. *N. diderrichii* is a forest species belonging to the family of Rubiaceae. Its regeneration *in situ* is laborious because of the dormancy of its seeds (Hawthorne, 1995a). Despite the abundance of fruits produced by trees every year, the regeneration by seed is very low. Under the mother-trees or far from them, no future stem had been identified on the field (Adjonou et al., 2014). In Togo, the overexploitation and lack of this species reforestation leads to its disappearance, as a result this species is classifies on list of the rare species (UNEP, 2010).

The *in situ* deficit of seedling is very worrisome when we know that the species is overexploited for its very resistant wood against the attacks of fungus (*Coriolus versicolor*), Lyctus, termites (*Reticulitermes santonensis*) and marine borers by local sawyers (Gérard et al., 1998; Soro et al., 2014). Furthermore, some of its organs (barks) are used in traditional medicines (Dibong et al., 2013). Its *in vitro* regeneration can be a good alternative to supply a stock of seedling of plantation (Pitekelabou et al., 2015). The study carried out here, is consisted to determine the efficiency of various sources of carbon on the rooting and the shoots development of seedlings, in order to identify the source of carbon adapted for *in vitro* micropropagation of *N. diderrichii.*

MATERIALS AND METHODS

Vegetal material used was constituted by uninodal fragment of seedlings stemming from *in vitro N. diderrichii*'s seeds germination. Culture is initiated on Woody plant medium (WPM) composed by Lloyd's and McCown's WPM macroelements (1980), 100 mg/L of myo-inositol, Murashiges' and Skoog's, microelements and vitamins (1962). This basic medium was alternately supplemented by each of the following carbohydrates: sucrose, maltose, lactose, glucose, fructose, galactose, sorbose, mannose, mannitol and sorbitol at 30 g/L rate. The ten culture media, differing by the source of carbohydrates, were solidified with agar-agar at 8 g/L. Their pH has been adjusted between 5.6 and 5.7 with NaOH at 1 N or HCl at 1 N.

These media were then distributed in tubes of 20 × 150 mm and sterilized at 120°C in autoclave under 1 bar during 20 min. Two months old *in vitro* plants were cut in uninodal fragments then put in culture on these ten media. Three months later, uninodal fragments cultivated in the presence of mannose or of mannitol, were transferred on a medium containing 30 g/L of sucrose. Tubes containing the *N. diderrichii's* explants are stored in a culture room with a photoperiod of 16 h at 27 ± 2°C and a light intensity of 120 $\mu Em^{-2}s^{-1}$. The light was supplied by fluorescent lamps.

Observations were performed during six weeks. At the end of every week, the number of roots, shoots, and nodes was noted and the shoots and roots length are measured. Every measure was made on a population of 20 individuals and has been repeated twice. The results presented in this work correspond to those obtained after six weeks of culture. Data were subjected to the variance analysis (one-way ANOVA) and means were classified in homogenous groups according to Newman and Keuls's range test ($\alpha = 0.05$) using Statistica version 10 (Statsoft Inc; Tulsa, USA: 2011) program.

RESULTS

Various carbohydrates used during this work gave diverse results. Sorbose showed a very noxious effect on the *N. diderrichii's* explants. Indeed none of them survived after 2 weeks of culture. Hundred per cent of necrosis was observed (Table 1). On galactose medium, 65% of explants sprouted, and no late evolution appeared except signs of necrosis. The bottom of explants took an orange color and leaves presented a typical yellowish aspect of senescence (Fiure 1). The use of sorbose or galactose caused sooner or later total necrosis of plants. On the other hand the use of mannose or mannitol has allowed 70% of explants to sprout but no late development of these sprouted explants was able to be observed (Figure 1).

Sucrose in culture medium allowed the best growth of roots which length (3.05^{d} ± 1.01 cm) is significantly different from that of plants' roots cultured in presence of all others carbohydrates used in present study (Table 2). It was noticed that in presence of sucrose, the shoots were bigger with longer internodes (Figure 1). Other carbohydrates: maltose, lactose, glucose, sorbitol and fructose, allowed the seedlings to produce roots, but did not favor a good growth of these. This low growth of the roots with length which was not more than 1.91^{a} ± 0.70 cm

Table 1. Effect of various carbohydrates on the viability and shoots development of *N. diderrichii*'s explants.

Sugar (30 g/L)	Necrosis explants (%)	No sprouted explants (%)	sprouted explants without growth
Saccharose	0[a]	0[a]	0[a]
Maltose	0[a]	0[a]	0[a]
Lactose	0[a]	0[a]	0[a]
Fructose	0[a]	0[a]	0[a]
Sorbose	100[c]	0[a]	0[a]
Glucose	0[a]	0[a]	0[a]
Galactose	25[b]	10[a]	65[b]
Mannose	0[a]	30[b]	70[b]
Sorbitol	0[a]	0[a]	0[a]
Mannitol	0[a]	0[a]	100[c]

Average ± standard deviation of the measures made on 20 explants and repeated twice. The values affected by the same letter in the same column, are not significantly different according to the test of Newman-Keuls in $P < 0.05$.

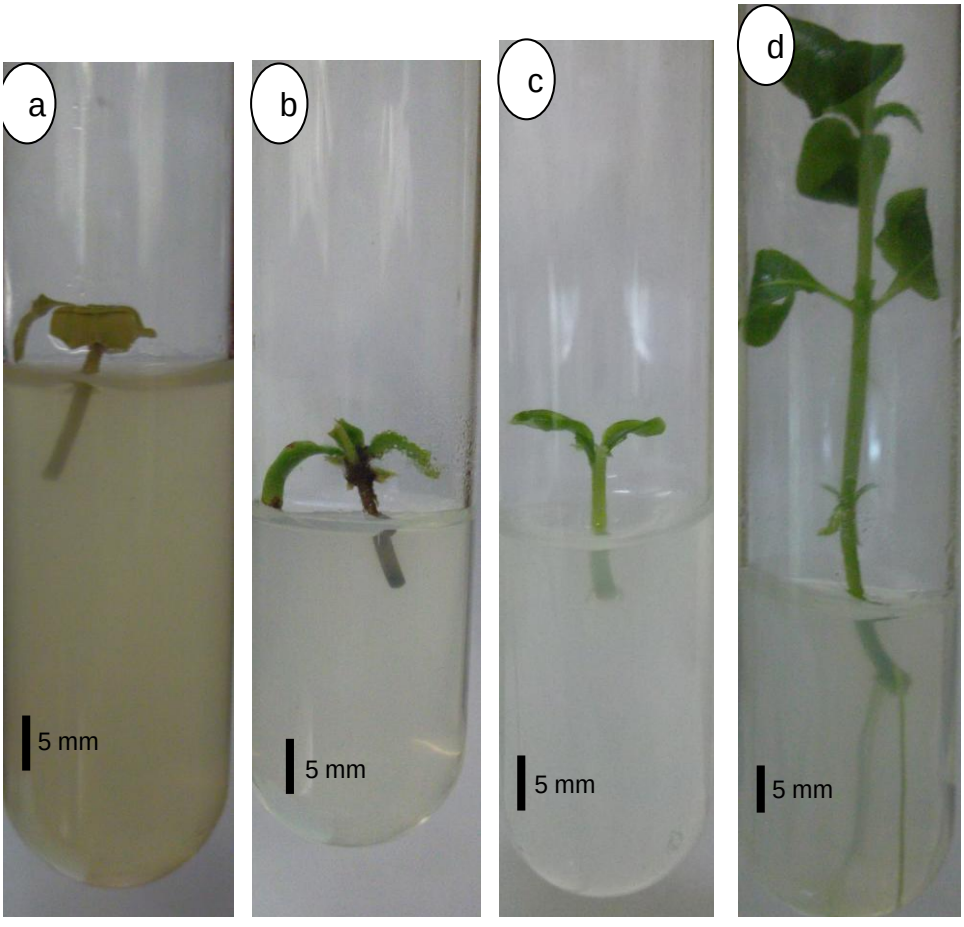

Figure 1. Development of seedlings on WPM medium containing different sugars. **(a)** Necrosis plant on sorbose. **(b)** Progressive necrosis plant on galactose. **(c)** Sprouted plant without growth on mannito. **(d)** Normal growth of plant on sucrose.

Table 2. Effect of various carbohydrates on rooting of the explants of *N. diderrichii.*

Carbohydrate (30 g/L)	Number of Roots / plant	Length of roots (cm)
Saccharose	$3.35^{a} \pm 1.18$	$3.05^{d} \pm 1.01$
Maltose	$3.20^{a} \pm 1.36$	$1.91^{a} \pm 0.70$
Lactose	$3.40^{a} \pm 1.19$	$1.41^{a} \pm 0.66$
Fructose	$3.80^{a} \pm 1.40$	$0.69^{c} \pm 0.73$
Sorbose	-	-
Glucose	$2.95^{ac} \pm 1.32$	$1.87^{a} \pm 1.01$
Galactose	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$
Mannose	$0.00^{b} \pm 0.00$	$0.00^{b} \pm 0.00$
Sorbitol	$2.35^{c} \pm 1.23$	$1.45^{a} \pm 0.66$
Mannitol	$0.50^{b} \pm 0.89$	$0.03^{b} \pm 0.05$

Average ± standard deviation of the measures made on 20 explants and repeated twice. The values affected by the same letter in the same column, are not significantly different according to the test of Newman-Keuls in $P < 0.05$.

Table 3. Effect of various carbohydrates on the development and the growth of the explants of *N. diderrichii.*

Carbohydrates (30g/L)	Nodes/plant	shoots/plant	Length of plant (cm)
Saccharose	$4.00^{c} \pm 1.56$	$2.30^{d} \pm 1.08$	$3.96^{c} \pm 1.71$
Maltose	$2.60^{e} \pm 0.94$	$1.25^{a} \pm 0.44$	$2.43^{b} \pm 0.84$
Lactose	$2,00^{d} \pm 0.79$	$1.15^{a} \pm 0.37$	$1.80^{b} \pm 0.75$
Fructose	$4.00^{c} \pm 0.86$	$1.90^{c} \pm 0.72$	$2.42^{b} \pm 0.85$
Sorbose	-	-	-
Glucose	$3.25^{f} \pm 0.97$	$1.55^{ac} \pm 0.60$	$2.18^{b} \pm 0.77$
Galactose	$0.65^{a} \pm 0.49$	$0.65^{b} \pm 0.49$	$0.07^{a} \pm 0.05$
Mannose	$0.70^{a} \pm 0.47$	$0.70^{b} \pm 0.47$	$0.07^{a} \pm 0.05$
Sorbitol	$1.45^{b} \pm 0.51$	$1.10^{a} \pm 0.31$	$0.60^{a} \pm 0.34$
Mannitol	$1.20^{ab} \pm 0.41$	$1.20^{a} \pm 0.41$	$0.10^{a} \pm 0.00$

Average ± standard deviation of the measures made on 20 explants and repeated twice. The values affected by the same letter in the same column, are not significantly different according to the test of Newman-Keuls in $P < 0.05$.

was even lower in fructose presence. Galactose or mannose in the medium inhibits completely rooting of *N. diderrichii's* plantlets and the addition of mannitol led to low rhizogenic plantlets ($0.50^{b} \pm 0.89$ root/plant) with null virtually growth ($0.03^{b} \pm 0.05$ cm) of shoots (Table 2).

Sucrose in culture medium allowed moreover the best growth of shoots which length ($3.96^{c} \pm 1.71$ cm) was significantly different from that of shoots cultured in presence of all others carbohydrates used. Indeed the length of plants obtained on medium supplemented with maltose, lactose, glucose, or fructose was not more than $2.43^{b} \pm 0.84$ cm when on medium supplemented with sorbitol, mannose, mannitol or galactose the plants' growth was virtually null ($\leq 0.60^{a} \pm 0.34$ cm) (Table 3). From all the sugars used in this work, sucrose gave greater shoots number ($2.30^{d} \pm 1.08$ shoots / plant). The greater multiplication rate which was 4.00 nodes / plant was obtained in this study in presence of sucrose or fructose, but the internodes of shoots obtained on fructose medium were very short and all of these nodes could not be recuperated for future multiplications.

Explants stemming from mannitol or mannose medium and transferred on medium of sucrose at 30 g/L gave the results carried in Table 4. The length of seedlings stemming from the medium containing sucrose is significantly different from the seedlings stemming from the medium containing mannose or mannitol. On the other hand, no significant difference was noted between the stemming

Table 4. Development of explants stemming from the first put in culture of six weeks in the presence of sucrose, mannose and mannitol and transferred on a medium containing sucrose.

Origin of explants	Roots/plant	Length of roots (cm)	Nodes/plant	Shoots/plant	Length of plant (cm)
Stemming from Sucrose	$3.35^{a} \pm 1.18$	$3.05^{a} \pm 1.01$	$4.00^{a} \pm 1.56$	$2.30^{a} \pm 1.08$	$3.96^{b} \pm 1.71$
Stemming from mannose	$1.35^{b} \pm 0.81$	$2.93^{a} \pm 1.43$	$4.45^{a} \pm 2.48$	$3.00^{a} \pm 1.84$	$2.81^{a} \pm 1.41$
Stemming from mannitol	$2.80^{a} \pm 0.95$	$2.98^{a} \pm 1.11$	$5.70^{a} \pm 3.34$	$3.55^{a} \pm 2.24$	$2.95^{a} \pm 1.21$

Average ± standard deviation of the measures made on 20 explants and repeated twice. The values affected by the same letter in the same column, are not significantly different according to the test of Newman-Keuls in $P < 0.05$.

seedlings of sucrose, mannose or mannitol concerning the average number of shoots, nodes and the roots length averages by plant. The multiplication rate was statistically the same for three types of origin of explants (Table 4).

DISCUSSION

The internal carbohydrate pool is suggested to have an important role in organogenesis of several woody species (Kromer and Gamian, 2000). However the exogenous supply of carbon sources can influence this organogenesis (De Neto and Otoni, 2003). The carbon sources serve as energy and osmotic agents to support the growth of plant tissues (Lipavska and Konradova, 2004). In present study, sucrose, maltose, lactose, fructose, glucose and sorbitol allowed all the root initiation of *N. diderrichii's* plantlets. However, according to these different sugars above, a significant difference was noticed on the growth of these different roots. Indeed, the length of initiated roots on the medium containing sucrose was significantly different from those obtained with others sugars used in this study. Sucrose, not only has been very favorable to the roots growth, but it has also favored the better growth of plantlets. The similar results were obtained for *Centell asiatica* L. (Anwar et al., 2005), *Pogostemon cablin* Berth (Swamy et al., 2010), *Solanum nigrum* Linn (Sridhar and Naidu, 2011). On the contrary, Preethi et al. (2011) observed that the fructose gave better results than sucrose, maltose and glucose used for the micropropagation of *Stevia rebaudiana*. For *Rosa rugosa*, Xing et al. (2010) showed that the action of the glucose was more favorable than sucrose for the proliferation and the growth of shoots and then the presence of sucrose in medium led to a yellowing of the leaves of plantlets. For peach rootstock GF 677 (Ahmad et al., 2007) and *Prunus armeniaca* (Marino et al., 1991), it was rather sorbitol which favored better efficient rooting and growth of plantlets than sucrose. On the other hand, Rahman et al. (2010) noted no significant difference when using sucrose, maltose and glucose in the *in vitro* culture of five varieties of potatoes. No sign of chlorosis was observed in the presence of sucrose in this work, while it provoked the chlorosis and the progressive death of shoots for *Prunus Mume* (Hisashi and Yasuhiro, 1996) and *Eclipta alba* (Baskaran and Jayabalan 2005). The growth and root initiation are highly energy requiring processes that can occur at the expense of available metabolic substrates, which are mainly carbohydrates (De Klerk and Calamar, 2002). Generally, sucrose is the carbohydrate used most of the time in the culture medium of tissues cultivated *in vitro* (Sul and Korban, 2004; Fuentes et al., 2000), because of the facilitated absorption of sucrose through the cellular membrane (Borkowska and Szezebra, 1991). In this work, among all sugars used, sucrose has been the one which has allowed obtaining not only a good rooting, but also a better growth of roots and plantlets. That suggests that the *N. diderrichii*'s plantlets absorb better sucrose and benefit from this sugar better energy than others sugars used here. The better growth of plantlets obtained in this work under effect of sucrose can be understand also in fact that this sugar in favoring the better growth of roots, favors the better absorption of nutritive substances in culture medium by plantlets. This reason has allowed to obtain the better shoots/plant and a good multiplication rate on this medium. According to Cuenca and Vieitez (2000), the ability to metabolize different types of carbohydrates differs within the plant kingdom. So the negative effects of some sugars on the plant growth are caused by their inefficiency metabolization by cells of these crops or by their reduced uptake in these plant species (Jain et al., 1997). These could be the main causes of a low growth of roots and plantlets of *N. diderrichii* on some media in this study. Galactose, mannose or mannitol in the culture medium inhibited the roots initiation partially or completely. The growth of plantlets was null in presence of these three carbohydrates. In galactose presence the plantlets finally necrotized. Similar result was found by Arditti and Ernst (1984) at orchids. In presence of mannose or mannitol, no plant was affected by necrosis, or developed any deformity. Sorbose has provoked total necrosis of all the explants of *N. diderrichii*. The result of these four types of sugar suggests that these sugars absorbed little or not and so provide few energy to *N. diderrichii* plantlets preventing them to initiate roots and have a good

development. However plants cultivated in the presence of the mannose or mannitol and transferred later on a medium containing sucrose developed well. No significant difference was noted between the rooting, the multiplication rate of these plants and those of the native plants of the medium containing sucrose. So the mannose or mannitol in the culture medium by slowing down the growth of *N. diderrichii*'s plants, can serve to keep this species' *in vitro* plants and a fast and massive multiplication can be done at the moment needed by transfer on the medium containing sucrose. A similar result was reported in the presence of mannitol for *Vanilla planifolia* where this carbohydrate slowed down the growth and allowed to keep this plant *in vitro* during seven years by making only a subculture / year (Divakaran et al., 2006). Charoensub and Phansiri (2004) also showed that the mannitol reduces the growth and the number of shoots by plant for *P. indica* Linn. According to the work of Da Silva and Scherwinski-Pereira (2011) with *Piper aduncum* and *P. hispidinervum*, the addition of the mannitol (1-3%) in the culture medium reduced the growth of seedlings, but with much higher contents, it causes the necrosis of seedlings. However the lethal concentration is dependent on the botanical species.

Conclusion

In the present study, observations showed that, the rooting and multiplication of shoots of *N. diderrichiis* seedlings *in vitro* are affected by the type of exogenous carbon source added to the medium. Among the different carbon sources used in this study, sucrose has been proved to be better for shoots' proliferation, growth and rooting of *N. diderrichii*'s seedlings, than other carbon sources in micropropagation. The divers effects obtained according to the carbohydrate in the culture medium confirm that a plant answers differently according to the carbohydrate as reported by other authors. It is thus imperative to adapt the protocol of micropropagation to every species by determining, in particular, the type of optimal carbohydrate to the development of the *in vitro* Plants' cultures. Besides, mannose and mannitol added in low concentration, in the culture medium slowed down and even stop the *N. diderrichii's* seedling growth without any later damage for these. The stabilizing action of these two carbohydrates on this botanical species' growth would allow to store and keep at a lower cost *in vitro* plants of *N. diderrichii*.

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Establishment of an efficient callus induction method from leaf and stem in kinnow mandarin (*Citrus reticulata* Blanco.) and citron (*Citrus medica* L.)

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Kinnow mandarin (*Citrus reticulata* Blanco.) is a highly adaptable variety among citrus cultivars in the climate of the region of Panjab, Pakistan and citron (*Citrus medica* L.) was the first of the citrus fruits known to become visible in the Mediterranean Basin. In Sylhet region of Bangladesh, it is commonly known as Zara lemon. The establishment of *in vitro* technique for seed germination and callus induction has been done for kinnow and citron in this experiment. In case of seed germination, ½ MS media supplemented with 6-benzylaminopurine (BAP) (0.5 mg/L)+ 1-naphthaleneacetic acid (NAA) (2.0 mg/L)+KIN (1.0 mg/L) shows best seed germination response (90%) for kinnow and BAP (1.0 mg/L)+NAA (0.5 mg/L) shows best (92%) for citron. For callus induction, 5 weeks old plantlets were used as a source of leaf and stem explants for both. For callus induction from leaf and stem of kinnow mandarin, 2,4-D (1.0 mg/L) shows best result (90%) for leaf while 2,4-dichlorophenoxyacetic acid (2,4-D) (1.0 mg/L)+BAP (0.5 mg/L)+NAA (0.25 mg/L) shows best callus response (95%) for stem explants. In case of citron, 2,4-D (1.0 mg/L) shows best callus response (80%) for both leaf and stem explants.

Key words: *In vitro,* kinnow mandarin, citron, seed germination, callus induction.

INTRODUCTION

Citrus is one of the leading tree fruit crop in the world. The genus citrus includes more than 162 species belonging to the family Rutaceae. Kinnow mandarin (*Citrus reticulata Blanco.*) is the most far and wide-planted mandarin hybrid in Pakistan. Kinnow has inherited heat tolerance from the cultivar King which helps it to survive in ruthless hot summer of Punjab. This "easy peel" citrus has assumed special economic importance and export demand due to its high juice content, special flavor, and as a rich source of vitamin C. Citron is a scented citrus fruit; it is a small tree about 2.44 to 4.57 m, having large fruit (20 to 22.5 cm long) that resembles pineapple in shape. It is botanically classified as *Citrus medica L.* and was the first of the citrus fruits to come into view in the Mediterranean region. In Sylhet region of Bangladesh it is locally known as Zara lemon and available in the local

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market. While the lemon or orange are peeled to consume their pulpy and juicy segments, the citron's pulp is dry, containing a small quantity of insipid juice, if any. The main content of a citron fruit is the thick white rind, which adheres to the segments, and cannot be separated from them easily. From ancient through medieval times, the citron was used mainly for medical purposes: especially to combat seasickness, pulmonary troubles, intestinal ailments, and other disorders. Citron juice with wine was considered as an effective antidote to poison. Because of the variability within the cultivar, the explants and the medium requirements are different, mentioned by various workers (Praveen et al., 2003; Singh et al., 2006; Gill et al., 1994; Jaskani et al., 2005; Bhatti et al., 2007).

The present investigation was undertaken to find out the suitable explant source along with the best suited concentration of plant growth regulators for callus induction and mass propagation of *C. reticulata* and *C. medica* through *in vitro.* Although, tissue culture and micro propagation protocols have been described for a number of citrus species and explants sources (Grinblat, 1972; Chaturvedi and Mitra, 1974; Barlass and Skene, 1982; Edriss and Burger, 1984; Duran Vila et al., 1989). Micro propagation has many advantages over conventional methods of vegetative propagation (cutting or seed) and its application in Horticulture, Agriculture and Forestry is currently expanding worldwide (Jeong et al., 1995). Embryogenic callus was successfully induced in six relatives of Citrus with combination of 5.0 mg/L BA, 2.5 mg 2,4-D and 600 mg/L malt extract in Murashige and Tucker (MT) medium (Jumin and Nito, 1995).

MATERIALS AND METHODS

The research project was conducted at the plant Genetic Engineering Laboratory, Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology (SUST), Sylhet-3114, Bangladesh. Mature seeds of kinnow mandarin and citron were germinated to produce young plantlets. 5 weeks old plantlets were then used as a source of explant for callus induction following callus induction, and were maintained in MS medium with supplementation of 2,4-D (1.0 mg/L).

Explant collection

Hundred seeds of kinnow mandarin and hundred seeds of citron were collected from Citrus Research Institute, Jointapur, Sylhet. Fresh healthy seeds of kinnow mandarin and citron were used as an explant for *in vitro* germination in half strength Murashige and Skoog (MS) regeneration medium. Callus development was done by using leaf and stem from germinated plantlets.

Sterilization of the explant

First of all, the seeds were dipped into 95% alcohol for 1 min. Then rinsed for four times with sterile distilled water. Afterwards, the seeds were dehusked and surface sterilized with 70% alcohol for 30 s. Furthermore, surface sterilization was done with 5% sodium Hypochlorite for 5 min. Then rinsed with sterile water for 3 to 4 times. In addition, it immersed into 2 to 3 drops of tween-20 for 15 min. Then washing of the explant took place with distilled water for 3 to 4 times. Before inoculation soaking of the explant was done on the filter paper.

Media for seed germination

Freshly collected 160 seeds of two varieties were cultured on half strength MS (Murashige and Skoog, 1962) basal medium enriched with hormonal concentration of BAP (1.0 mg/L)+NAA (0.5 mg/L) , BAP (0.5 mg/L)+NAA (2.0 mg/L)+KIN (1.0 mg/L), 2,4-D (2.0 mg/L)+BAP (1.0 mg/L)+NAA (0.5 mg/L), and ½ MS medium without growth regulator. Approximately, 10 seeds were cultured in each hormonal combination on separate test tube for seed germination and each combination has two replications in a separate time frame (a gap between 15 days). pH of the media was adjusted to 5.8 to 6.0. Visual observation was taken for each replication with 10 samples for every 7 days and effect of hormonal combination on seed germination was recorded.

Media for callus induction

Five weeks old leaf and stem were used for callus induction in the case of kinnow mandarin and citron. Leaf and stem cuttings of kinnow mandarin and citron plantlet cultured on MS (Murashige and Skoog, 1962) basal medium supplemented with hormonal concentration of 2,4-D (1.0, 1.5, 2.0, 4.0) mg/L alone and 2,4-D (1.0 mg/L)+BAP (0.5 mg/L)+NAA (0.25 mg/L) in combination. For callus proliferation media with 2, 4-D (1.0 mg/L) was used. In every case 3% sucrose was added. pH of the media was adjusted to 5.8 to 6.0. Visual observation was taken every 7 days and effect of different treatment was quantified on the basis of percentage of callus induction.

Explant inoculation

Each explant was inoculated in test-tube by the help of a sterile forceps in the laminar air flow chamber. After the inoculation of explants, the test-tubes were transferred to the culture room and incubated at 25°C. The photoperiod was maintained as 16 h light and 8 h darkness by 40 W white florescent tubes light with intensity from 2000 to 3000 lux. Data was recorded every week for three months.

Statistical analysis

All the data were recorded at regular interval for analysis and reckoned under statistical basis. Arithmetic mean (A.M.) and standard deviation (S.D.) were evaluated by analyzing data with Microsoft excel 2007. Standard error (S.E.) was calculated by dividing standard deviation by square root of the total 20 replication for a single variety in each hormonal concentration. In case of our experiment error related to contamination was calculated properly and expected values were taken from the calculation.

RESULTS AND DISCUSSION

Direct regeneration from embryonic seed

Citrus seeds have a very short life because they are amenable to injured by drying during storage and thus

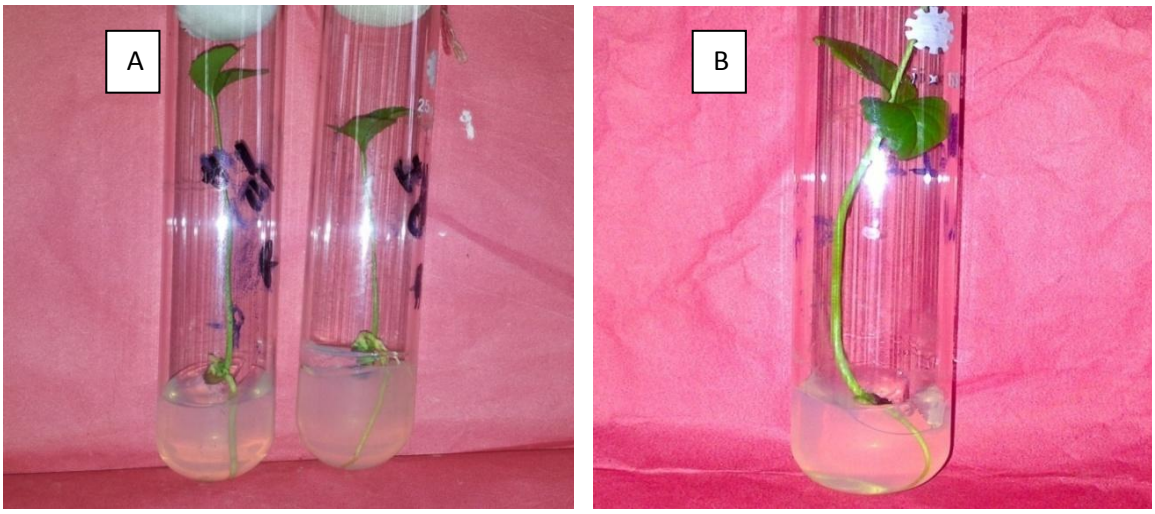

Figure 1. Plantlets were obtained using BAP (1.0 mg/L)+NAA (0.5 mg/L) for kinnow mandarin (A) and citron (B).

Figure 2. Callus of kinnow on ½ MS medium supplemented with 2, 4- D (2.0 mg/l) +BAP (1.0 mg/l) +NAA (0.5 mg/l).

lose their viability (Johnston, 1968). This is why freshly isolated seeds from kinnow and citron were used. Removal of seed coat showed an early response for shoot formation. In case of sweet orange, highest (70%) shoot formation was obtained from seeds without seed coat (Azim et al., 2011). In this experiment direct regeneration from seed was done for obtaining 5 weeks old plantlets of kinnow and citron species. Half strength MS media supplemented with BAP (0.5 mg/L)+NAA (2.0 mg/L)+KIN (1.0 mg/L) shows (90%) germination in case of kinnow mandarin and in case of citron media supplemented with BAP (1.0 mg/L) +NAA (0.5 mg/L) shows (92%) best result for germination of plantlets (Figure 1). Altaf et al. (2009) reported that, the seeds formed callus in MS medium supplemented with BA and 2,4-D each at 1 mg/L. In our experiment, in hormonal combination of 2,4-D (2.0 mg/L) +BAP (1.0 mg/L)+NAA (0.5 mg/L) citron shows 70% seed germination response, where seed callus found for kinnow mandarin (Figure 2). This implies that, citron may not require BAP and NAA along with 2,4-D in this concentration for callus induction from their seed thus callus induction requires different concentration of 2,4-D and other plant hormones for citron (Table 1).

Table 1. Effect of different hormonal combination for seed germination after 5 weeks of observation (20 replications for each combination for a single species).

Species	Treatments (mg/L)	Growth rate	Average germinated plant height for 20 replications (cm)	Average leaf number of all the replica	Seed Germination percentage (%)
Kinnow mandarin	BAP(1.0)+NAA(0.5)	Average	2	2±1	85
	BAP(0.5)+NAA(2.0)+KIN(1.0)	Good	2.5	3±1	90
	2,4-D(2.0)+BAP(1.0)+NAA(0.5) [Callus obtained]	---	----	----	----
	MS basal	Low	1.5	2±1	70
Citron	BAP(1.0)+NAA(0.5)	Excellent	3.0	3 ±1	92
	BAP(0.5)+NAA(2.0)+KIN(1.0)	Good	2.5	2±1	85
	2,4 D(2.0) +BAP(1.0)+NAA(0.5)	Average	1.5	2±0	75
	MS basal	Average	2	2±1	80

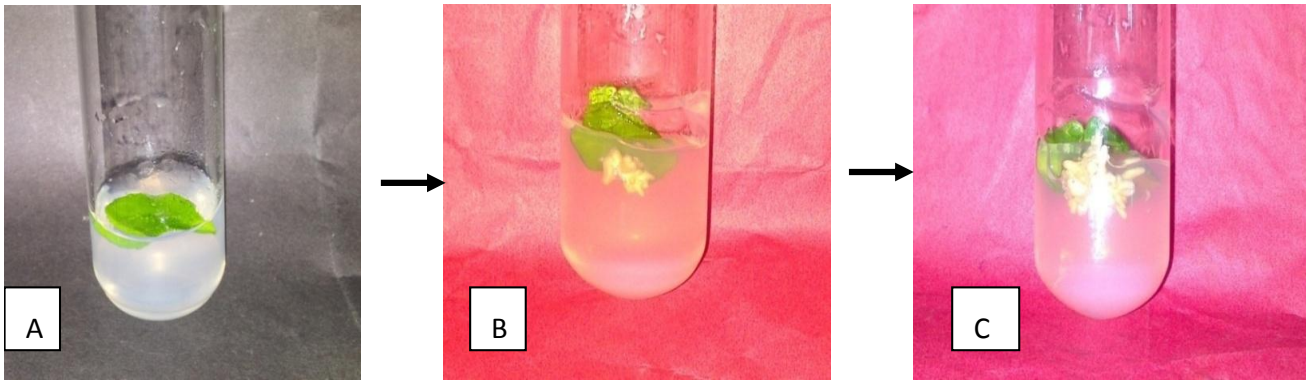

Figure 3. Callus induction from kinnow leaf in 2,4-D (1.0 mg/l). A = Initial stage after inoculation; B = callus after 21 days; C = Callus after 45 day.

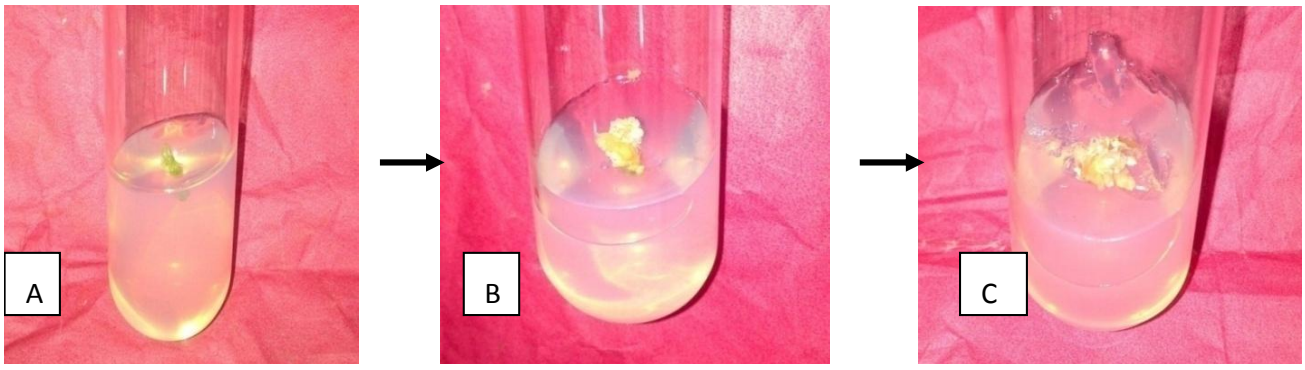

Figure 4. Callus induction from kinnow stem in 2, 4-D (1.0 mg/l) +BAP (0.5 mg/l)+NAA(0.25 mg/l). A = Initial stage; B = callus after 21 days; C = callus after 45 days.

Callus induction from leaf and stem of kinnow mandarin and citron

In this experiment, callus induction was done from leaf and stem explants that were taken from 5 weeks old plantlets. After 4 to 6 weeks of inoculation high efficiency callus was produced (Figures 3, 4, 5 and 6). Altaf et al. (2009) reported that hormonal combination for good callus induction for seedling leaf of kinnow mandarin is BA+ GA (each at 1 mg/L) + 2,4-D at 0.5 mg/L + proline at

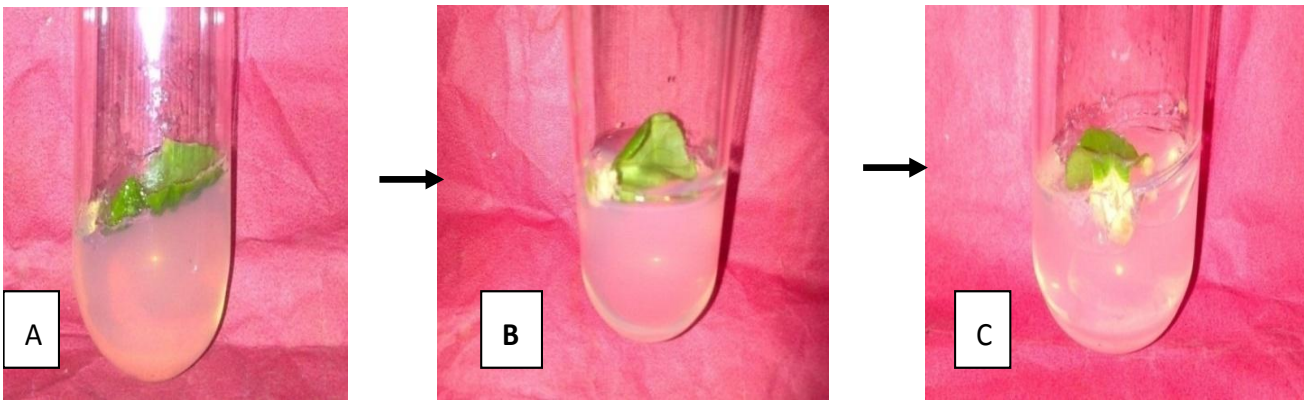

Figure 5. Callus induction from citron leaf in 2,4-D (1.0 mg/l). A = Initial stage after inoculation; B = callus after 30 days; C = callus after 45 day.

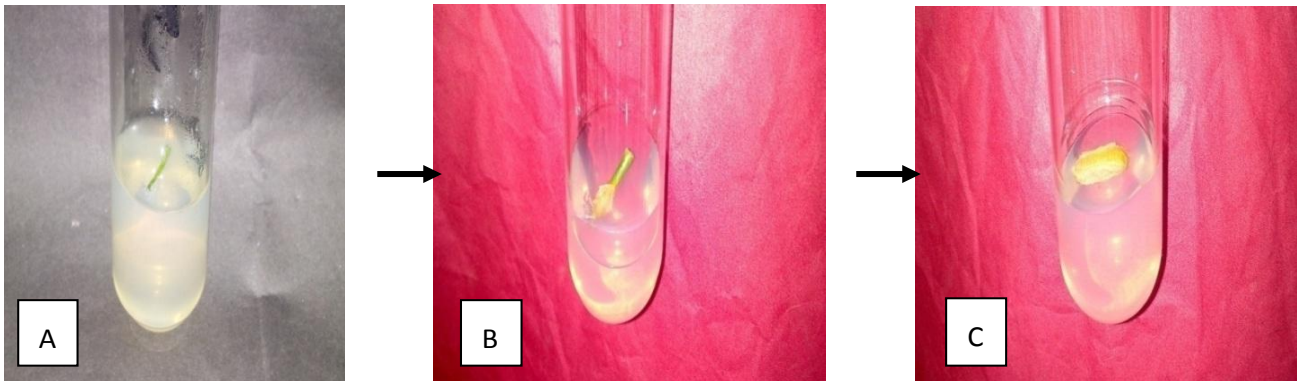

Figure 6. Callus induction from citron stem in 2,4-D (1.0 mg/l). A = Initial stage after inoculation; B = callus after 30 days; C = callus after 45 days.

Table 2. Effect of different hormonal combination for callus induction from leaf and stem explant of kinnow mandarin, after 45 days of observation (total of 20 replications for each combination).

Explant for callus	Treatments (mg/L)	Average number of calli	Color of callus	Average callus type
Leaf	2,4-D(1.0)	7±2	Yellowish whitish	Nodular compact
	2,4-D(1.5)	5±1	Yellowish white	Nodular compact
	2,4-D(2.0)	3±1	Whitish	Smooth compact
	2,4-D(4.0)	2±2	Whitish	Smooth compact
	2,4-D(1.0)+BAP(0.5)+NAA(0.25)	2±1	Whitish	Smooth compact
Stem	2,4-D(1.0)	3±2	Yellowish white	Nodular compact
	2,4-D(1.5)	2±1	Yellowish white	Nodular compact
	2,4-D(2.0)	5±1	Yellowish white	Nodular compact
	2,4-D(4.0)	2±2	Whitish	Nodular compact
	2,4-D(1.0)+BAP(0.5)+NAA(0.25)	6±2	Yellowish white	Nodular compact

5 mg/L. So, in our experiment callus induction has been done for both leaf and stem with a little adjustment of BAP and NAA along with 2,4-D and good callus performance has been found for stem rather than leaf. Approximately 90% of the callus was nodular compact, while 10% was smooth and compact in case of kinnow (Table 2). The color of the callus produced was whitish and yellowish white. The lower concentration of 2,4-D

Table 3. Effect of different hormonal combination for callus induction from leaf and stem explant of Citron after 45 days of observation (Total 20 replications for each combination).

Explant for callus	Treatments (mg/L)	Average number of calli	Color of callus	Type
Leaf	2,4-D(1.0)	4 ±2	Yellowish whitish	Nodular compact
	2,4-D(1.5)	3 ±1	Yellowish white	Nodular compact
	2,4-D(2.0)	2 ±2	Yellowish white	Smooth compact
	2,4-D(4.0)	2 ±1	Whitish	Smooth compact
	2,4-D(1.0)+BAP (0.5)+NAA (0.25)	1 ±1	Whitish	Smooth compact
Stem	2,4-D(1.0)	N/A	Yellowish white	Smooth compact
	2,4-D(1.5)	N/A	Yellowish white	Smooth compact
	2,4-D(2.0)	N/A	Yellowish white	Smooth compact
	2,4-D(4.0)	N/A	Whitish	Smooth compact
	2,4-D(1.0)+BAP (0.5)+NAA (0.25)	N/A	Yellowish white	Smooth compact

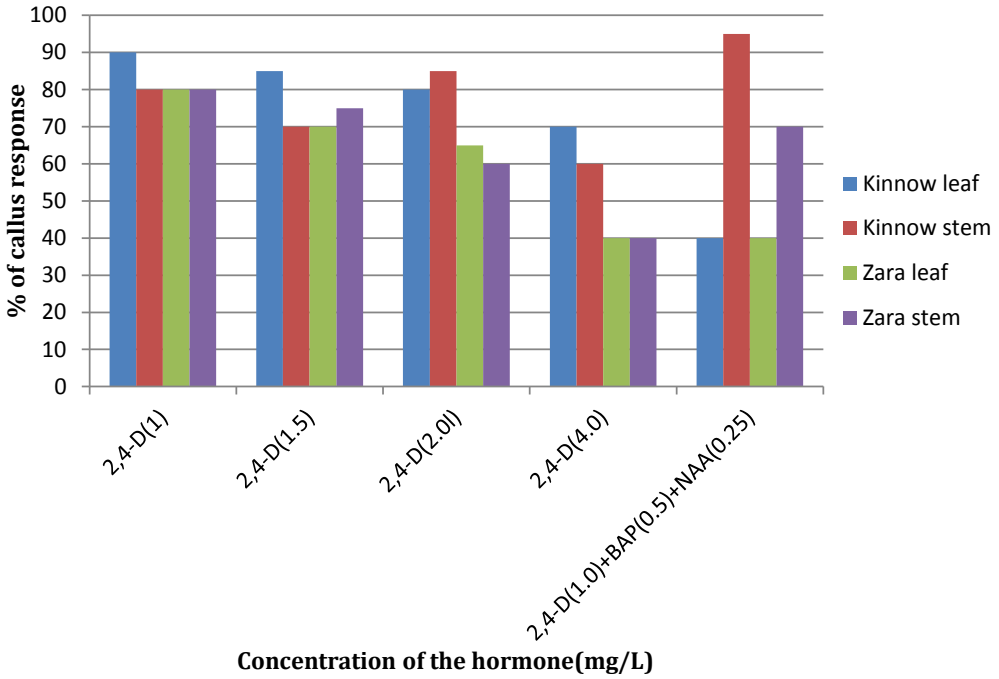

Figure 7. Effects of different hormonal combination in callus induction from leaf and stem of Kinnow mandarin and citron (callus response shown in percentage after 45 days of observation on a total 20 replication per hormonal combination).

(1 mg/L) was sufficient to induce callus in 96% of cultures from nodal segments whereas for leaf segments higher concentration of 2,4-D (4 mg/L) was to be used to achieve 98% callus induction (Savita et al., 2010). Different experiment shows that callus induction depended on explant type as well as concentration and type of plant growth regulator used, for example *Albizzia lebbek* (Lakshmana Rao and De, 1987); *Lonicera japonica* (Georges et al., 1993) and *Holarrhena antidysenterica* (Raha et al., 2003). In this experiment, approximately 40% of the callus of citron was nodular compact, while 60% was smooth and compact. The color of the callus produced was whitish and yellowish white (Table 3). For leaf, maximum callus were nodular and thus no of calli was countable and but for stem callus were smooth. By comparison with kinnow mandarin, hormonal combination of 2,4-D with BAP and NAA is less efficient for callus induction in citron (Figure 7).

In case of callus induction from leaf of kinnow mandarin 2,4-D (1.0 mg/L) shows (90%) best result and 2,4-D (1.0 mg/L)+BAP (0.5 mg/L)+NAA (0.25 mg/L) shows best (95%) for stem. In case of callus induction of Citron 2, 4-D (1.0 mg/L) shows (80%) best result for both leaf and stem. Thus, we can consider media with hormonal

combination of 2,4-D (1.0 mg/L) for efficient callus induction from leaf and stem of both citrus cultivar.

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Full Length Research Paper

Synthesis and characterization of 2-mercapto-N-methyl imidazole substituted benzimidazole derivatives and investigation of their effect on production of plantlets in *Oncidium* Gower Ramsey

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A series of benzimidazole derivatives (5a-c) were synthesized by coupling 5-substituted 2-chloromethyl benzimidazole (3a-c) with 2-mercapto-N-methyl imidazole. The synthesized compounds were characterized by infra-red (IR), nuclear magnetic resonance (NMR) and elemental analyses. Further, the synthesized compounds were tested on plantlet production from protocorm like bodies (PLBs) sections of *Oncidium*. PLBs sections were cultured on half strength modified Murashige and Skoogs (MS) medium alone and also modified MS medium supplemented with the synthesized chloro, methyl and nitro derivatives individually at 2 or 5 µM concentrations. Among these three compounds, PLBs sections cultured on medium supplemented with nitro compound (5c) at 5 µM concentration produced maximum number (95) of plantlets.

Key words: Benzimidazole derivatives, protocorm like bodies, plant growth regulators, 2-mercapto-N-methyl imidazole.

INTRODUCTION

The presence of imidazole and benzimidazole moieties in biological systems such as histidine, vitamin-B_{12} etc. has proven their biological relevance (Sorrell, 1989). Thus, these compounds have been extensively studied for anticancer (Swiatkiewicz et al., 2014), antibacterial (Zhang et al., 2009), anti-trypanosomatid (Boiani et al., 2009), antimicrobial (Jardosh et al., 2013), antitumor (Hranjec et al., 2010), fungicide (Wena et al., 2013) and other biological activities. Reports are available where imidazole and benzimidazole derivatives have been

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#Equal contribution

Abbreviations: PLBs, Protocorm like bodies; **CMNP,** 5-chloro-3-methyl-4-nitro-1H-pyrazole; **ZnAAC,** zinc-amino acid complexes; **TIBA,** 2,3,5-triiodobenzoic acid; **PCIB,** 2-(p-chlorophenoxy)-2-methylpropionic acid; **IR,** infra-red; **NMR,** nuclear magnetic resonance.

tested even for plant growth regulation (Lacova et al., 1993; Cavender, 1986). PLBs of orchids are the versatile system to study the effect of plant growth regulators on plant morphogenesis. *Oncidium* Gower Ramsey is commercially an important ornamental orchid. *In vitro* production of somatic embryos/PLBs from explants of *Oncidium* are controlled by several factors including nature of explants, sugar source, macro and micro nutrients, vitamins, amino acids, and plant growth regulators (Chen and Chang, 2001; 2002; 2003a; 2003b; 2004; Su et al., 2006). Apart from auxins, cytikinins, gibberellin, abscisic acid, ethylene, jasmonic acid, Brassinosteroids, salicylic acid and polyamines, there are several reports on the synthesis of other novel compounds, which are regulating the plant growth and development. Phenylurea derivatives [N,N-bis-(2,3-methylenedioxyphenyl)urea (2,3-MDPU)] have been found to enhance production of root system in *Capparis spinosa* (Carra et al., 2012). Cytokinin-like activity was exhibited by the urea derivatives but their activity was less compared to kinetin and N-phenyl-N′-(4-pyridyl) urea (Yonova and Stoilkova, 2004). The compound, in which the un-substituted phenylcarbamoyl group was directly attached to the piperazine ring, also showed cytokinin-like activity and significantly stimulated betacyanin synthesis in *Amaranthus caudatus* (Stoilkova et al., 2014). Adventitious shoot proliferation was enhanced in *Spathiphyllum floribundum* with the addition of imidazole and paclobutrazol individually to medium containing cytokinin (Werbrouck and Debergh, 1996). *In vitro* shoot and root growth in wheat and sorghum was inhibited by 3-aryl-1*H*-indazoles (Chattha et al., 2012).

The 5-chloro-3-methyl-4-nitro-1H-pyrazole (CMNP), a pyrazole-derived plant growth regulator promoted starch degradation and senescence-related symptoms in *Arabidopsis* (Alferez et al., 2007). Zinc-amino acid complexes (ZnAAC) have been shown to stimulate the root and shoot growth in *Lactuca sativa* (Ghasemi et al., 2013). A derivative of benzimidazole like 5-hydroxybenzimidazole (Hoyle and Robin, 2009) helps in increasing shoots growth, root growth, leaf area. Literature survey indicates that very little attention has been paid to the investigation of the benzimidazole derivatives (5a-c) for their effect on production of plantlets in *Oncidium* Gower Ramsey. In the present investigation, 2-mercapto-N-methyl imidazole substituted benzimidazole derivatives were synthesized, characterized and studied for their effects on production of plantlets from PLBs sections of *Oncidium*.

MATERIALS AND METHODS

All the chemicals used in the present study were procured from Sigma–Aldrich, USA. The synthesized compounds were confirmed by elemental and spectral analyses. ^{1}H NMR spectra were recorded on a Bruker 400 MHz multinuclear spectrometer with TMS as internal standard (chemical shift in δ ppm) and are given in Figures 5 to 7. Mass specta were recorded on a LC/MSD-Trap-XCT. C, H, N, S analyses were carried out using a Carlo-Erba analyzer. IR spectra were recorded on a Bruker IR spectrometer after grinding the sample with KBr.

Plant growth regulation study

Oncidium Gower Ramsey plants were procured from Indo-American Seeds Pvt. Ltd. Bangalore, India and maintained in Biotechnology department, R V College of Engineering, Bangalore. The PLBs were produced from the nodal explants of inflorescence on Murashige and Skoog (MS, 1962) medium with modification [½ strength MS salts, MS vitamins, 1 g/l tryptone, 20 g/l sucrose, 1 g/L charcoal, 65 g/L potato tubers, 8 g/l agar, and 5 µM of thidiazuron (TDZ)]. The *in vitro* produced PLBs were maintained on modified MS medium free from TDZ. The pH of media was adjusted to 5.8 before solidifying with agar (Himedia, India). Media were dispensed to culture bottles and sterilized at 121°C for 20 min. The thermoliable compounds such as TDZ, vitamins and glycine were filter sterilized using 0.45 µm membrane filters (Sartorious, Germany) and added to autoclaved media. The sections of PLBs were cultured on modified MS medium supplemented with chloro, methyl and nitro derivatives individually at 2 or 5 µM concentrations. The cultures were maintained under controlled environmental conditions (22 ± 2°C temperature with 16/8 h photoperiod). Five PLBs sections were placed in each culture bottle, three replicates were maintained for each concentration, and each experiment repeated twice. Cultures were observed and morphological changes such as number of explants responded and number of shoots developed from explants were recorded. One-way analysis of variance (ANOVA) was performed using SPSS software and means were compared with Duncun's multiple range test (Duncan, 1955).

Experimental

Synthesis of the compounds (3a-c)

To a solution of 4-substituted benzene-1,2-diamine (1.0 mmol) in 4N HCl (10 ml), chloroacetic acid (2.0 mmol) in 4 N HCl (7 ml) was added. The reaction mixture was refluxed for 4 h. The completion of reaction was monitored by TLC. The reaction mixture was cooled to room temperature then it is basified with Sodium bicarbonate. The precipitate so obtained was filtered, dried and recrystallized with ethanol. Characterization of the compound was done by NMR and liquid chromatography mass spectrometry (LCMS). Compounds were directly taken to next step without purification. NMR and LCMS data complies with literature data (Satyanarayana and Nagasundara, 2007).

Synthesis of the compounds (5a-c)

The compounds were synthesized by the following common procedure. A mixture of 2-mercapto-N-methyl imidazole (1 mmol) and sodium hydroxide (1 mmol) in methanol (10 mL) was stirred for about 30 min. A methanol solution (10 mL) of 5(6)-substituted 2-chloromethyl benzimidazole (1 mmol) was added slowly with stirring and the mixture was refluxed for about 8 h. The progress of the reaction was monitored by TLC and after completion; reaction mixture was quenched with water and extracted with ethyl acetate. The organic layer was washed with water and brine, finally dried with anhydrous sodium sulphate. The organic layer was concentrated and the resulting crude compound was purified by column chromatography over silica gel (60 to 120 mesh) using ethyl acetate (100%). Synthesis of above benzimidazole derivatives 5(a-c) is given in Figure 1. Synthesis of 6(5)-Chloro-2-((1-methyl-1*H*-

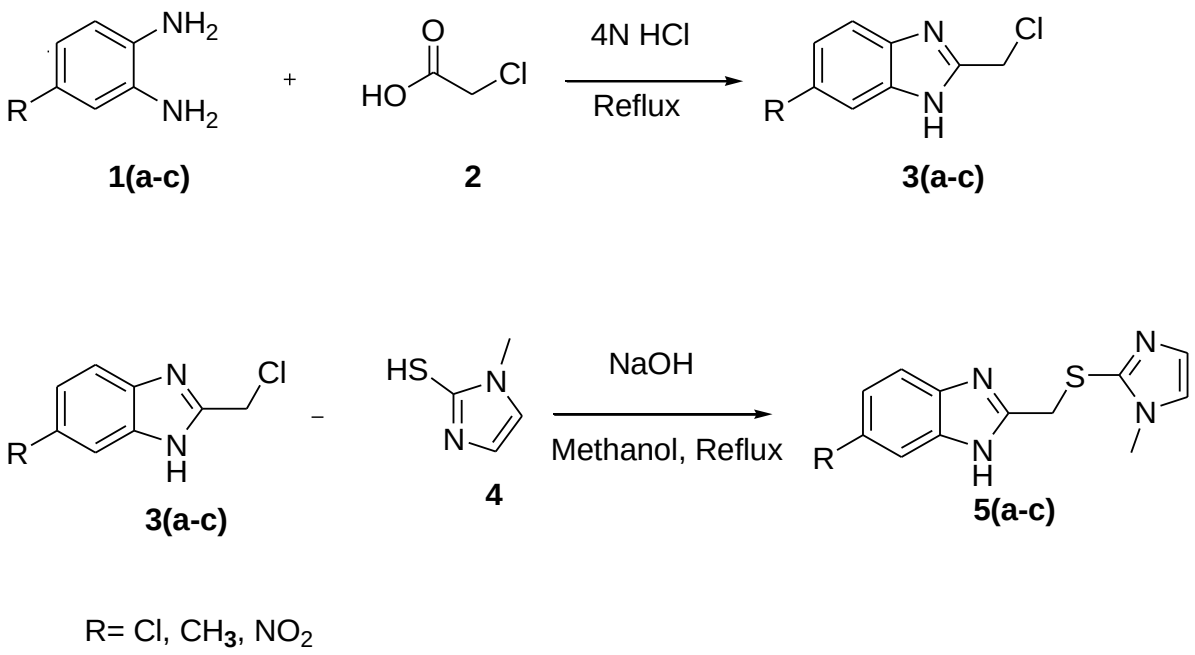

Figure 1. Synthesis of 2-mercapto-N-methyl imidazole substituted benzimidazole derivatives (5a-c).

imidazol-2-ylthio)methyl)-1*H*-benzimidazole (5a) Compound (5a) was synthesized from 2-mercapto-N-methyl imidazole (0.114 g, 1 mmol) and 6-chloro-2-(chloromethyl)-1*H*-benzimidazole (0.201 g, 1 mmol). Physical data for 5a: Yield: 85%, m.p: 130 to 132°C, Analysis for $C_{12}H_{11}ClN_4S$: found: C (51.28), H (3.98), N (20.73), S (10.86). Calculated % C (51.70), H (3.98), N (20.10), S (11.50). ^{1}H NMR ($CDCl_3$, 400 MHz): 3.49 (s,3H,N-CH_3), 4.39 (s,2H, -CH_2), 6.98 (s,1H,Ar-H), 7.41 to 7.17 (m,1H,Ar-H), 7.26 (s,1H,Ar-H), 7.50 (d,1H, *J* = 12.0Hz, Ar-H), 7.56 (d,1H, *J* = 4.0 Hz Ar-H). ^{13}C NMR ∂(ppm): 31.53, 32.90, 121.96, 123.79, 126.08, 128.77, 138.84, 152.53, MS (ESI) m/z: 279.06, 281.06 (M+2), IR (KBr, cm^{-1}): 3657, 3383, 1625, 1341, 1229, 853.

Synthesis of 6(5)-Methyl-2-((1-methyl-1H-imidazol-2-ylthio)methyl)-1H-benzimidazole (5b)

Compound (5b) was synthesized from 2-mercapto-N-methyl imidazole (0.114 g, 1 mmol) and 2-(chloromethyl)-6-methyl-1*H*-benzimidazole (0.181 g, 1 mmol). Physical data for 5b: Yield: 62%, m.p: 62 to 64°C, Analysis for $C_{13}H_{14}N_4S$: found % C (60.19), H (5.05), N (21.88), S (11.98); calculated % C (60.44), H(5.46) N (21.69), S (12.41); ^{1}H NMR ($DMSOd_6$, 400 MHz): 3.59 (s,3H,N-CH_3), 4.41 (s,2H,-CH_2), 2.45 (s, 3H, Ar-CH_3), 6.96 (s,1H,Ar-H), 7.03 (d,1H, *J*=8.08Hz, Ar-H), 7.15 (s,1H, Ar-H), 7.35 (s,1H,Ar-H), 7.45 (d,1H, *J*=8.2Hz, Ar-H). ^{13}C NMR ∂(ppm): 29.66, 31.00, 33.48, 122.60, 123.97, 128.73, 132.34, 142.54, 151.97. MS (ESI) m/z: 259.10; IR (KBr, cm^{-1}): 3664, 3144, 1704, 1314, 1265, 865.

Synthesis of 2-((1-Methyl-1H-imidazol-2-ylthio)methyl)-6-nitro-1H-benzimidazole (5c)

Compound (5c) was synthesized from 2-mercapto-N-methyl imidazole (0.114 g, 1 mmol) and 2-(chloromethyl)-6-nitro-*1H*-benzimidazole (0.211 g, 1 mmol). Physical data for 5c: Yield: 55%, m.p: 134 to 136°C, Analysis for $C_{12}H_{11}N_5O_2S$: found %: C (49.11), H (3.35), N (23.92), S (10.75); calculated % C (49.82), H (3.83), N (24.21), S (11.08); ^{1}H NMR ($DMSOd_6$, 400 MHz): 3.50 (s,3H,N-CH_3), 4.46 (s,2H,-CH_2), 6.96 (s,1H,Ar-H), 7.25 (s,1H,Ar-H), 7.67 (d,1H, *J*=12Hz, Ar-H), 8.09 to 8.06 (m,1H,Ar-H), 8.41 (s,1H,Ar-H). ^{13}C NMR ∂(ppm): 31.45, 32.93, 123.87, 128.85, 138.61, 142.49. MS (ESI) m/z: 290; IR (KBr, cm^{-1}): 3603, 3244, 1712, 1322, 1277, 866. The structural and spectroscopic assignments were made according to the reported literature (Shivakumaraiah et al., 2003; Sahin et al., 2002).

RESULTS AND DISCUSSION

IR spectra

The compounds 5(a-c) exhibit a broad band around 3244 to 3383 due to ν(NH) of the Benzimidazole ring and band in the region at 1625 to 1704 assignable to υ(C=C) and υ(C=N). All the three compounds exhibit characteristic band around 1400 cm^{-1} due to N-CH_3 stretching vibration.

NMR Spectra

The ^{1}H NMR spectra of the heterocycles (5a-c) exhibit a singlet in the range of δ 4.39 to 4.46 due to -CH_2-protons. The N-CH_3 proton signals were observed in the range of δ 3.49 to 3.60. Compound 5b exhibited signals due to aromatic protons in the range δ 7.15 to 7.47, whereas, in the corresponding chloro and nitro derivatives (5a and 5c), these signals were observed in the range δ 7.26 to 8.41. The downfield shift in the aromatic

Table 1. Effects of compounds 5(a-c) on plantlet regeneration from sections of *Oncidium* PLBs*.

Treatment	Concentration (µM)	No. of plantlets per culture bottle•
Control		41[f]
5a	2	56[e]
	5	84[b]
5b	2	69[d]
	5	84[b]
5c	2	76[c]
	5	95[a]

Control: modified MS medium (1/2 strength MS salts, MS vitamins, 1 g/l tryptone, 20 g/l sucrose, 1g/l charcoal, 65 g/l potato tubers, 8 g/l agar). *Fifteen (15) PLBs sections were cultured for each treatment (five PLBs sections per culture bottle. •Means followed by same letters are not significantly different according to DMRT at P= 0.05 (Duncan, 1955)

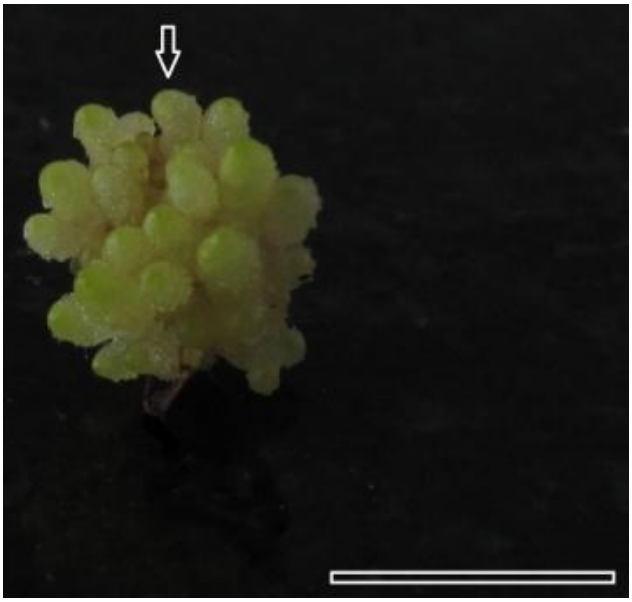

Figure 2. Induction of globular embryos from PLBs sections of *Oncidium* on modified MS medium supplemented with 5 µM compound **5c** (bar = 0.5 cm).

proton signals in the case of 5a and 5c is attributed to the deshielding effect of -Cl and -NO_2 groups on the ring. The number of protons calculated from the integration of ^{1}H NMR spectra is in accordance with C, H, N, S analysis of the above compounds.

Effect of 2-mercapto-N-methyl imidazole substituted benzimidazole derivatives on plantlet production

PLBs sections of *Oncidium* cultured on modified MS medium alone and also on modified MS containing chloro, methyl and nitro derivatives at 2 or 5 µM concentrations showed significant difference in production of plantlets (Table 1). After two-weeks of culture, PLBs sections were swollen, and in another two weeks it produced globular-embryo-like structures (Figure 2). Globular embryo-like-structures were developed into cotyledonary-embryo-like structures (Figure 3). On the same medium, these cotyledonary-embryo-like-structures developed into plantlets (Figure 4). PLBs explants produced average

Figure 3. Development of cotyledonary stage embryos of *Oncidium* on modified MS medium containing 5 µM compound 5c (bar = 1.4 cm).

Figure 4. Development of plantlets on modified MS medium containing 5 µM compound 5c (bar = 3 cm).

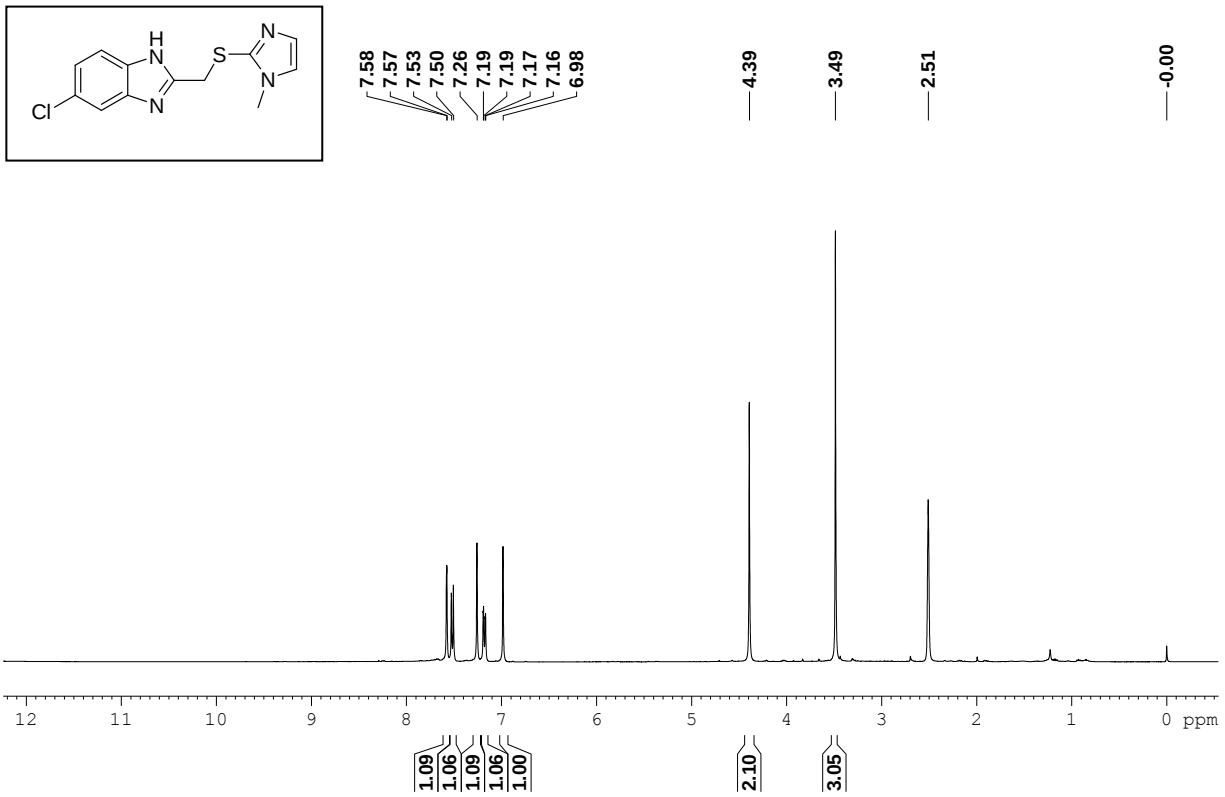

Figure 5. [1]H NMR spectrum of compound 5a.

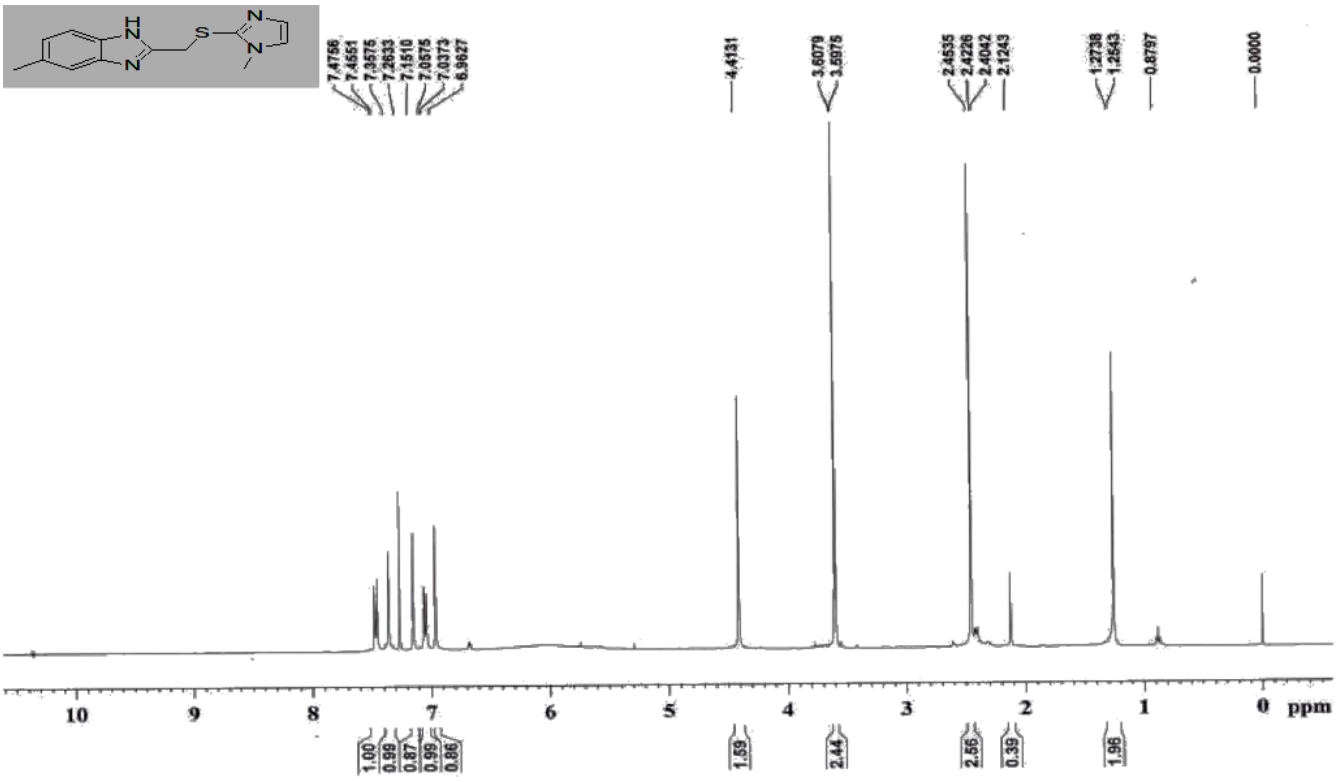

Figure 6. [1]H NMR spectrum of compound 5b.

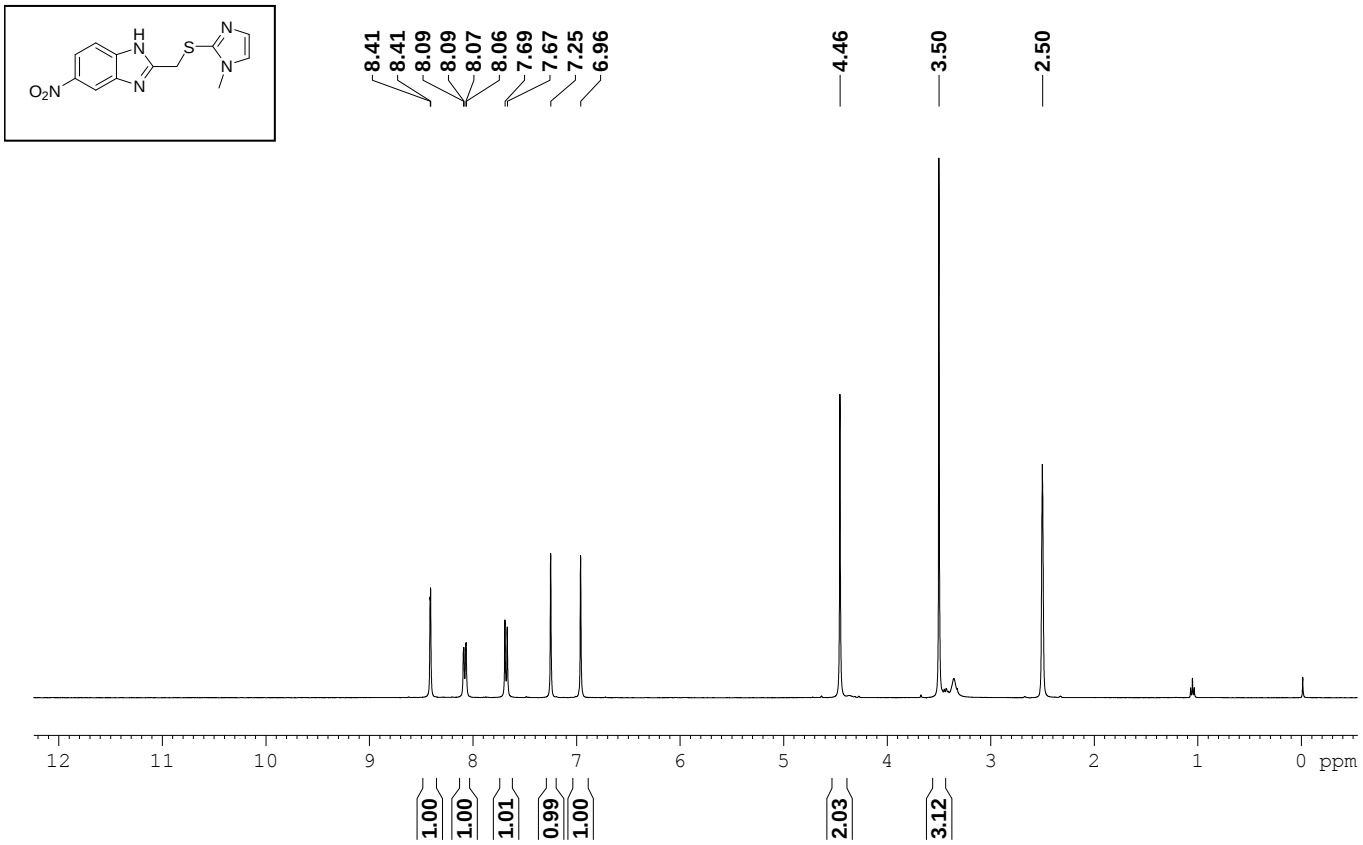

Figure 7. ^{1}H NMR spectrum of compound 5c.

number (41) of plantlets on modified MS medium alone. The production plantlets from PLBs explants were enhanced significantly with the addition of chloro, methyl or nitro derivatives to the modified MS medium. Chen and Chang (2001; 2003a; 2003b; 2004) studied the effects of plant growth regulators such as auxins, cytokinins, gibberellins (GA_3), ancymidol, cycocel, paclobutrazol, 2,3,5-triiodobenzoic acid (TIBA), 2-(p-chlorophenoxy)-2-methylpropionic acid (PCIB) on production of somatic embryos and subsequent development of plantlets in *Oncidium*. In present study, PLBs sections produced 56, 69 and 76 numbers of plantlets on medium supplemented with 2 µM of chloro, methyl and nitro derivatives, respectively (Table 1).

Direct somatic embryogenesis was reported in *Oncidium* Gower Ramsey from leaf explants (Chen and Chang, 2001). Similarly, in present investigation, plantlets were produced directly from the PLBs sections through somatic embryogenesis without intervening callus phase. Among three compounds studied in the present investigation, explants cultured on medium supplemented with 5 µM nitro derivatives produced maximum number (95) of plantlets (Table 1). The involvement of nitro radical in the nitro derivative in a wide range of plant functions such as growth senescence, fruit ripening and responses to adverse environmental conditions (Sanchez B-Calvo et al., 2013) is expected to be the reason for its higher activity when compared to chloro and methyl derivatives

Conclusions

The analytical data and spectral data confirm the formation of the compounds (5a-c). Production of plantlets from PLBs section of *Oncidium* was improved with the addition of chloro or methyl or nitro derivatives at 2 or 5 µM concentrations to the induction medium. Medium supplemented with 5 µM nitro derivative exhibited maximum number of plantlets.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Production of lignocellulolytic enzymes from three white-rot fungi by solid-state fermentation and mathematical modeling

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Three species of white rot-fungi (*Pleurotus ostreatus*, *Coriolus versicolor*, and *Lentinula edodes*) were grown on 12 solid media based on several lignocellulosic materials (oak sawdust, coconut husks, coffee husks and corn bran) during 49 days. The media had varied carbon/nitrogen ratios and $CuSO_4$ content. The objective of the work was to evaluate the effect of the media formulation on the production of lignocellulolytic enzymes and degradation of lignocellulosic components by the three fungal species. *C. versicolor* exhibited the highest ability to degrade the three main polymers of the lignocellulosic waste materials employed and to produce ligninases with titers as high as 107 U/g solid substrate in the case of laccase. In addition, a mathematical model describing the fermentation kinetics of the cell biomass growth, degradation of lignocellulosic components, and lignocellulolytic enzyme production for the fungal species/medium combination exhibiting the best performance under solid-state fermentation conditions was proposed and validated in the case of *C. versicolor*. The mathematical model could be used to provide valuable information on the process itself as well as to contribute to the development of a future commercial process for lignocellulolytic enzyme production.

Key words: White-rot fungi, cellulases, ligninases, degradation of lignocellulosic materials, fermentation kinetics.

INTRODUCTION

The lignocellulolytic enzymes have a wide spectrum of applications in several industrial sectors. These enzymes are being used for breakdown of lignocellulosic materials (particularly, agricultural and agro-industrial wastes) into fermentable sugars, which can be converted into valuable products like ethanol, lactic acid and butanol. In particular, cellulases are currently used at pilot and semi-industrial plants during the hydrolysis of cellulose derived from lignocellulosic biomass for fuel ethanol production (Sánchez and Montoya, 2012). Cellulolytic enzymes are also

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employed for improvement of paper texture, biodeinking during paper recycling, biopolishing of the fabrics, and detergent production. In the same way, ligninases are used for delignification of forage grass and agricultural wastes for animal feed (Ruqayyah et al., 2013). Ligninolytic enzymes are also used to decolorize synthetic dyes contained in wastewater from textile industry (Grassi et al., 2011). In fact, ligninases are being involved in the transformation and mineralization of organopollutants with lignin-like structures, so they have been proposed to treat and degrade polychlorinated biphenyl pesticides, polycyclic aromatic hydrocarbons, effluents from bleaching mills and synthetic polymers, among others (Pointing, 2001).

The so-called white-rot fungi synthesize and secrete into the media based on lignocellulosic materials (liquid or solid) an important amount of cellulases, xylanases, and ligninases. For this reason, the cultivation of these fungi under conditions of submerged or solid-state fermentation is being intensively studied. Basidiomycetes like *Pleurotus ostreatus* (Carabajal et al., 2012), *Coriolus versicolor*, and *Lentinula edodes* (Elisashvili et al., 2008) produce the above-mentioned enzymes using different lignocellulosic feedstocks, especially agro-industrial wastes. The enzymes released by these fungi synergistically act on the lignocellulosic complex in order to degrade it and generate the sugars and other compounds needed for their development. The cellulose biodegradation implies the initial action of the endoglucanases (ENG) and exo-cellobiohydrolases (also called exoglucanases, EXG), which attack the amorphous and crystalline structure of the cellulose forming cellotrioses and cellobiose. These oligosaccharides are later converted into glucose by the β-glucosidase. The hemicellulose (xylan) hydrolysis requires the action of the endoxylanase (ENX) and β-xylosidase, although other enzymes that hydrolyze the substituted xylans are needed to completely degrade this polysaccharide. The lignin is broken down by a non-hydrolytic mechanism through the interaction of different extracellular enzymes, mainly phenoloxidases like laccase (LAC), and peroxidases like manganese peroxidase (MnP) and lignin peroxidase.

The production of lignocellulolytic enzymes by white-rot fungi depends on several different factors. In particular, the micro-environmental and nutritional conditions in the substrate (that is, lignocellulosic materials) directly affect the growth rate of each one of the fungal species employed. Thus, the composition of the media on which these fungi are cultivated plays a crucial role during the synthesis of lignocellulolytic enzymes. In particular, the titers of oxidoreductases such laccase and manganese peroxidase are influenced by the concentration of metals like copper in the culture medium. Although it is not a common fact that the manganese peroxidase is induced with a metal other than manganese, Mouso et al. (2003) already has shown that copper and cadmium induce the synthesis of this enzyme in *Stereum hirsutum*, and Levin et al. (2002) have demonstrated that laccase and manganese peroxidase production is enhanced by adding $CuSO_4$ to the medium in the case of *Trametes trogii*. On the other hand, the carbon/nitrogen (C/N) ratio is an important factor when white-rot fungi are cultivated to produce lignocellulolytic enzymes, especially under solid-state fermentation (SSF) conditions. For instance, Bento et al. (2014) pointed out that an appropriate C/N ratio during solid-state cultivation of macromycetes like *L. edodes* is critical to achieve the complete colonization of the substrate and suitable levels of ligninolytic activity. Therefore, the selection of the solid materials (substrates) and the determination of the medium composition are crucial to attain a significant amount of active lingo-cellulolytic enzymes.

The mathematical modeling of the cultivation process has a paramount importance in order to develop an efficient fermentation process at pilot scale or industrial level. Several mathematical expressions have been proposed to describe the fungal growth. These models involve linear or logistic expressions for growth rate. However, these models do not include the effect of the nutrient concentration on the fungal development (Ikasari and Mitchell, 2000; van de Lagemaat and Pyle, 2005). Likewise, Monod-type and modified logistic expressions have been employed to describe the biomass growth for different fermentation types (Mitchell et al., 2004; Tavares et al., 2005). The mathematical expressions used to model the product formation depend on the type of metabolite synthesized by the fungus. For primary metabolites, the models are simple since a directly proportional relationship between growth and product formation rates is generally assumed. The biosynthesis of intermediary and secondary metabolites is much more complex to describe because there exist no well-defined simple expressions covering the formation of these compounds. Currently, there are not available mathematical models allowing the description not only of the growth of macromycetes fungi, but also of the biosynthesis of enzymes obtained from them during SSF. This difficulty is related to the reduced knowledge on the combined influence of factors like the physical characteristics and chemical composition of the solid media employed, environmental conditions, features of the metabolic pathways involved, and presence of inhibitors, among others.

The objective of this work was to evaluate the effect of the carbon/nitrogen ratio and copper sulfate (II) concentration on production of lignocellulolytic enzymes by three white-rot fungi species (*P. ostreatus*. *C. versicolor* and *L. edodes*) grown on 12 different solid media based on lignocellulosic substrates. In addition, this work was aimed at proposing and validating a mathematical model describing the fermentation kinetics of the cell biomass growth, degradation of lignocellulosic components, and lignocellulolytic enzyme production for the fungus/medium combination exhibiting the best

Table 1. Carbon/nitrogen (C/N) ratios and copper sulfate (II) concentrations employed for formulations of twelve solid media.

Formulation	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
$CuSO_4$ (wt.%)	0.16	0.08	0.16	0.08	0.16	0.08	0.16	0.08	0.16	0.08	0.16	0.08
C/N ratio	142.6	89.7	114.6	93.6	100.9	91.8	85.3	84.9	55.1	51.6	117.1	111.0

The solid media contained (dry basis) 40% oak sawdust, 20% coconut husks, and 2% soybean oil. In addition, the media contained coffee husks and corn bran, which were used to adjust the C/N ratio.

performance under SSF conditions.

MATERIALS AND METHODS

Microorganisms

Three species of basidiomycetes were employed for production of lignocellulolytic enzymes by SSF. These species are deposited at Culture Collection of Macrofungi at Universidad de Caldas (Manizales, Colombia). *L. edodes* CICL54 strain was donated to this collection by the National Coffee Research Center, Cenicafé, located in Chinchiná (Colombia). *C. versicolor* PSUWC430 strain was acquired to the Pennsylvania State University (USA). *P. ostreatus* UCC001 strain belongs to the collection. The species were maintained on potato dextrose agar (PDA) at 4°C with periodic transfer.

Spawn production

The three fungal species were adapted by inoculating wet wheat grains with five pieces of 1.0 cm^2 of the mycelium extended on PDA until complete colonization. Spawn of all the species evaluated was prepared on wheat grains previously cleaned and hydrated until 40% moisture content. The wheat grains were packed in bi-oriented polypropylene bags. Each bag of 20 cm diameter and 30 cm height contained 1 kg of solid substrate. One square hole with 2.54 cm side was made at the top of each bag and covered with a microporous breather strip to allow for gas exchange. The hydrated grains contained in the bags were tyndallized at 121°C for 30 min. Then the grains were aseptically inoculated with 4% (wet basis) of each fungal species previously adapted as described above and incubated for 12-15 days at 25°C until complete colonization.

Culture media

Twelve (12) different formulations of the solid media for SSF named F1, F2,..., F12 were employed. These media were formulated on dry basis with 40% oak sawdust, 20% coconut husks and 2% soybean oil as fixed components. The concentration of these fixed components was defined in a previous work (Montoya, 2012). The remaining components (coffee husks and corn bran) were varied in order to modify the C/N ratio as shown in Table 1. The concentration of copper sulfate (II) was varied on two levels: 0.16 wt.% for odd formulations and 0.08 wt.% for even formulations. All media were formulated at 60% moisture. The media were packed in bi-oriented polypropylene bags with 200 g solid material and tyndallized at 121°C for 30 min. Then the media were inoculated with 4% (wet basis) spawn related to the solid mass in laminar flow chamber, and incubated at 25°C under dim light for 49 days. Fifteen (15) samples were taken (two per week); for each sample, three replicas from different bags cultivated under the same conditions were performed.

Carbon/nitrogen ratio was determined based on the measurement of the organic matter in the 12 formulations using the method reported by Walkley and Black (1934), which employs concentrated sulfuric acid and a potassium dichromate solution. The sample is read at 585 nm. The total carbon content corresponds to the 58% organic matter. The total organic nitrogen was determined by Kjeldahl method (Kjeldahl, 1883).

Quantitative determination of enzymatic activities

Extracts for the determination of enzyme activities were obtained from 1 g fresh substrate in 12 mL sterile neutral distilled water. Then the resulting suspension was sonicated for 5 min and stirred for 10 min, with subsequent filtration and centrifugation.

Cellulolytic and xylanolytic activities

Endo-1,4-β-*D*-glucanase (EC 3.2.1.4): A reaction between 0.5% carboxymethyl cellulose (CMC) in sodium acetate buffer solution with a pH of 4.8 and 100 µL enzymatic extract is performed for 30 min at 50°C. The enzymatic reaction was stopped by adding 3,5-dinitrosalicilic acid (DNS). Immediately after this, the same DNS added is used for determination of reducing sugars according to the method of Miller (1959) for which the absorbance is read at 540 nm. One unit of enzyme activity (U) was defined as the amount of enzyme that produces 1 µmol reducing sugars per minute. A calibration curve of reducing sugars constructed from different glucose concentrations was used to quantify the enzyme activity.

Exo-1,4-β-*D*-glucanase (EC 3.2.1.91): A reaction between 1% crystalline cellulose in sodium acetate buffer solution with a pH of 4.8 and 100 µL enzymatic extract is performed for 60 min at 50°C. The enzymatic reaction was finished by adding DNS. Immediately after this, the same DNS added is used for determination of reducing sugars according to the method of Miller (1959). The samples were centrifuged before reading the absorbance at 540 nm. One unit of enzyme activity (U) was defined as the amount of enzyme that releases 1 µmol reducing sugars per minute.

β-glucosidase (EC 3.2.1.21): A reaction between 0.02% *p*-nitrophenyl β-*D*-glucopyranoside (the substrate) in sodium acetate buffer solution with a pH of 4.8 with 100 µL enzymatic extract is performed for 30 min at 50°C. The enzymatic reaction was finished by adding Clark and Lubs buffer solution with pH of 9.8 and reading the absorbance at 430 nm (Wood and Bhat, 1988). One unit of enzyme activity (U) was defined as the amount of enzyme that produces 1 µmol *p*-nitrophenol per minute. A calibration curve was constructed using *p*-nitrophenol in order to quantify the enzyme activity.

Endo-1,4-β-*D*-xylanase (EC 3.2.1.8): A reaction between 0.2% xylan in sodium acetate buffer solution with pH of 4.8 and 100 µL enzymatic extract is performed for 30 min at 50°C. The enzymatic reaction was stopped by adding DNS. Immediately after this, the same DNS added is used for determination of reducing sugars according to the method of Miller (1959) for which the absorbance is read at 540 nm. One unit of enzyme activity (U) was defined as

the amount of enzyme that produces 1 µmol reducing sugars per minute. In this case, the calibration curve was constructed using xylose.

Ligninolytic activities

Laccase (EC 1.10.3.2): A reaction between 0.5 mM ABTS (2.2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate)) solution in 0.1 M sodium acetate buffer with a pH of 3.6 and the enzymatic extract was performed according to the method of Paszczynski and Crawford (1991). The absorbance was read at 420 nm after 3 min of reaction at 30°C, time needed for color change from transparent to turquoise green. One unit of enzyme activity (U) was defined as the amount of enzyme required to oxidize 1 µmol ABTS in 1 min.

Manganese peroxidase (EC 1.11.1.13): The substrates of the enzymatic reaction were 0.01% phenol red in 0.1 M sodium succinate buffer solution with a pH of 4.5 and manganese sulfate (0.22 g/L) with 0.2 mM hydrogen peroxide as initiator. After 10 min of reaction of these substrates with the enzymatic extract, the process is finished by adding 5 N NaOH in order to read the absorbance increase at 610 nm (Paszczynski et al., 1988). One unit of enzyme activity (U) was defined as the amount of enzyme needed to oxidize 1 µmol red phenol in 1 min.

Chemical analyses

During the incubation of each solid medium (substrate), 15 samples were collected, two per week, until the 49th day for each one of the three fungal species (*C. versicolor*, *P. ostreatus* and *L. edodes*). The samples were dried at 101°C until constant weight, ground and stored for determination of chitin content. The content of fungal biomass in the solid media was indirectly estimated based on the determination of the structural component of chitin, the N-acetyl-*D*-glucosamine (NAGA), after hydrolysis with 6 N HCl according to the method of Plassard et al. (1982). The NAGA content per gram of mycelium for each one of the three species grown in liquid medium was determined in a parallel way; for this, 250 mL flasks containing 100 mL liquid medium were used. The liquid medium employed for these purposes had the following composition (in g/L): glucose, 30; yeast extract, 6; $MgSO_4.5H_2O$, 0.5 and $CaCl_2$, 0.1. The NAGA content for the mycelium grown in liquid medium was determined according to the method of Plassard et al. (1982) as well.

The fiber components (cellulose, hemicellulose and lignin) for each one of the formulations of the solid media and for all the 15 samples collected during the cultivation of each fungal species, as well as the soluble fraction, were determined by using the results of the determination of neutral detergent fiber, acid detergent fiber, and acid detergent lignin. To determine these fiber types, each dried and ground solid sample underwent three hydrolysis in series during 70 min each: i) hydrolysis with sodium lauryl sulfate and others; ii) hydrolysis in ammonium bromide in 1 N sulfuric acid solution; and iii) hydrolysis with 72% (w/v) sulfuric acid. At the end of each hydrolysis, samples were washed and dried at 105°C until constant weight (Leterme, 2010). The content of reducing sugars as glucose was determined by the DNS method (Miller, 1959).

Experimental design

In order to find the most appropriate combination of the three fungal species and 12 medium formulations having the best performance in terms of lignocellulolytic enzyme production and degradation of lignocellulosic components of the substrates, a randomized bi-factorial experimental design was used with three levels for the first factor (fungal species) and 12 levels for the second factor (formulation of the solid medium). The formulations (F1 to F12) varied their C/N ratio and the copper sulfate (II) concentration as shown in Table 1. The response variables were six enzyme activities (ENG, EXG, BG, ENX, LAC, and MnP), and the fiber components (content of cellulose, hemicellulose, and lignin in the samples). The response variables for each fungus/formulation combination were measured for 15 incubation times during the fermentation process. Thus, three different experimental series were performed (one series for each fungal species) by varying the medium formulation. Each sample was taken by triplicate from different bags cultivated under the same conditions. All the statistical analysis for this work were performed by using the software Matlab® 2010b (MathWorks, USA). The analyses were carried out in three steps. Firstly, an analysis of variance (ANOVA) was done for all the data obtained during the experimental design considering all the fungus/formulation combinations with 5% significance level. For this, the anovan Matlab function was applied. Then a comparative Kruskal Wallis analysis for degradation of cellulose, hemicellulose and lignin was performed for each one of the three fungal species studied by using the kruskalwallis and multcompare functions. Once the degradation maximum was determined for each species, a new comparative analysis to find the combination with the best performance in terms of fiber degradation at the end of fermentation (49th day) was carried out.

Mathematical modeling of fungal growth and enzyme production

Matlab® was also used to solve the mathematical models proposed in this work. For this, the ode45 function based on an explicit Runge-Kutta (4,5) formula using a Dormand-Prince pair as well as the ode15s function based on a variable-order formula employing numerical differentiation were applied. The model parameters were determined by non-linear regression from the experimental data obtained by using the nlincon and Fmincon Matlab functions.

RESULTS

Effect of C/N ratio and cupric sulfate on degradation of lignocellulosic wastes and enzyme production

The interval of C/N ratio used for the 12 medium formulations was from 50 to 140 with a variation in the nitrogen content ranging from 0.29 to 0.8% in dry basis. All the three fungal species were able to grow on all the media formulations and to produce significant enzyme activities for all the C/N ratios during 49 incubation days. All the nitrogen contents in the formulations stimulated the macromycetes growth during the vegetative phase increasing the biomass growth and cellulose consumption as observed in Figure 1 (all formulations are not presented due to space constraints). It is worthy to point out that the variation of nitrogen content and carbon source was ensured employing the same materials (oak sawdust, coconut husks, coffee husks, corn bran, and soybean oil) and not modifying the nitrogen and carbon sources. The ANOVA performed considering the lingocellulolytic enzymes titers exhibited during the SSF (Figure 2) demonstrated the variability in the seven enzyme activities measured depending on the species, formulation and incubation time. The first ANOVA indicated that there exist significant differences within each factor

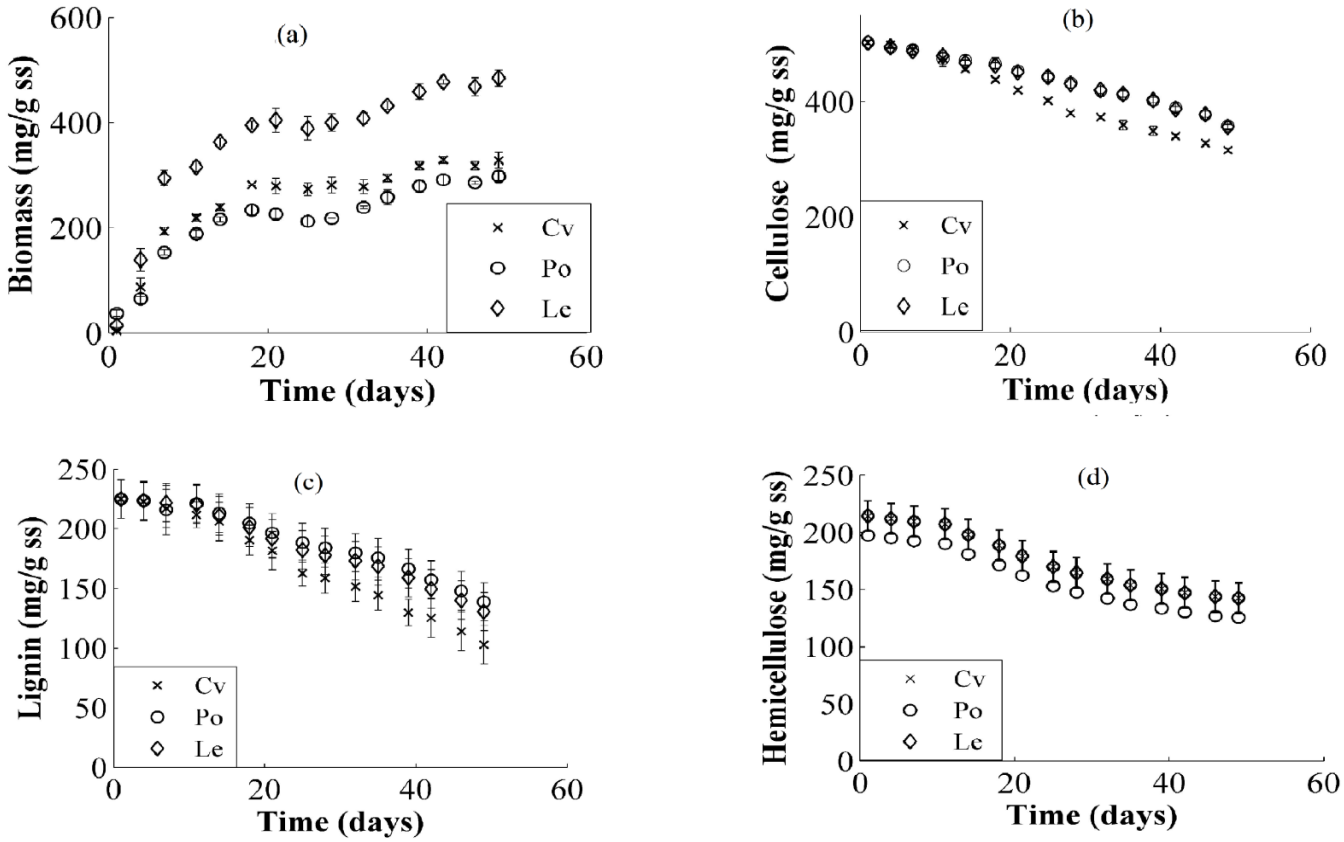

Figure 1. Biomass production and cellulose consumption for *C. versicolor* (Cv), *P. ostreatus* (Po), and *L. edodes* (Le) grown on different solid media based on oak sawdust, coconut husks, coffee husks, corn bran, and soybean oil during 49 cultivation days under SSF conditions. **(a)** Biomass growth on F1 medium. **(b)** Cellulose consumption for F12 medium. **(c)** Lignin degradation for F3 medium. **(d)** Hemicellulose consumption for F7 medium. The formulation for each medium is deciphered in Table 1.

(species and formulation) and between them (5% significance level). Then the maximum lignocellulolytic enzyme activities for each fungal species were determined graphically considering the 15 incubation times sampled as presented in Table 2. For instance, the highest EXG levels were obtained for *L. edodes* grown on F6 medium at 21st day (106.44 U/g solid substrate), while the lowest EXG corresponded to *P. ostreatus* grown on F12 medium at 35th day (44.05 U/g solid substrate).

As observed in Figure 1, fungal biomass increased for all the formulations during 49 days of fermentation. In the case of the hydrolases (ENG, EXG, BG and EXN), the variability of the enzymatic activities found is as wide among the three fungal species and 12 media formulations, that there exists no clear evidence that the cupric sulfate amounts added to the substrates (0.08% and 0.16%) cause neither inhibition nor direct activation of the fungal ability to produce these enzymes.

Time course of enzyme production during SSF

The time course of enzyme production measured during 49-day SSF did not present a defined tendency for any fungus or medium formulation. Nevertheless, from all the experimental data obtained in this work, an overall trend consisting in the decrease of enzymes production after a specific incubation time can be observed. ENG and EXG tend to decrease after 20 days of incubation, in coincidence with the moment when the three species have completed the colonization of the solid medium (substrate). However, some combinations showed a different behavior, as in the case of *P. ostreatus* grown on F3 medium for which the ENG presented a stable trend until the 35th day with a decrease until the end of the process. In the case of EXG for all the fungi, the F7–F10 formulations presented an increase in enzyme activities until the 30th day with a decrease until the end of the measurements (Figure 2b). On the other hand, EXN activity was more disperse than in the case of the two foregoing hydrolases although values as high as 90 U/g ss (solid substrate) for F5 medium were achieved for *C. versicolor*. Regarding the BG activity, there is no clear trend of the data, but a decrease in the activity with the time is noticeable for all fungus/formulation combinations. The two oxidoreductases studied in this work exhibited different behaviors. The LAC activity considerably varies

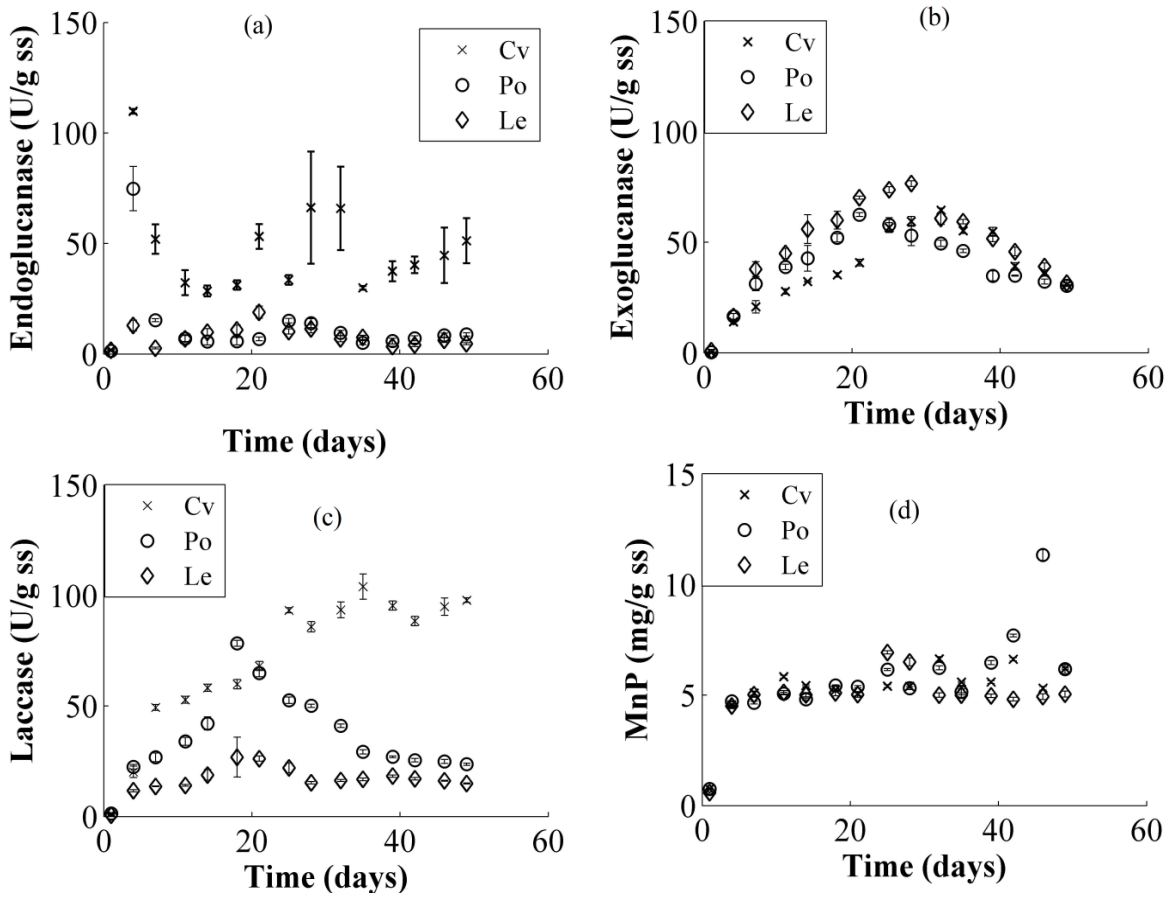

Figure 2. Time course of lignocellulolytic enzyme production by *C. versicolor* (Cv), *P. ostreatus* (Po), and *L. edodes* (Le) grown on different solid media based on oak sawdust, coconut husks, coffee husks, corn bran, and soybean oil during 49 cultivation days under SSF conditions. **(a)** Endoglucanase acitvity (ENG) for F6 medium. **(b)** Exoglucanase activity (EXG) for F10 medium. **(c)** Laccase activity (LAC) for F9 medium. **(d)** Manganese peroxidase activity (MnP) for F4 medium. The formulation for each medium is deciphered in Table 1.

among the combinations, being *C. versicolor* the species with highest titers of this enzyme, which were in the range 80-100 U/g ss for some formulations (Figure 2c). In contrast, the MnP activity was generally low for the time interval of the measurements (Figure 2d).

Degradation of lignocellulosic components

In order to choose the fungus/formulation combination with the best performance regarding the degradation of the main components of the lignocellulosic wastes (cellulose, hemicellulose and lignin) caused by the release of an important amount of lignocellulolytic enzymes, several ANOVAs were performed. The results of the first ANOVA showed that there exist significant differences among the treatments for each fungal species as mentioned above. For each fungus, the maximum of each enzyme activity was determined. Then a comparative analysis of these maxima was carried out using the multcompare Matlab function and applying a Kruskal-Wallis test for each activity. In this way, significant differences were found among the maxima of enzyme activity.

To choose the best fungus/formulation combination, a new comparative analysis of the degradation percentages of the fiber components was done. For this, the results of degradation percentages for each fungus and formulation at the end of SFF (49^{th} day) were analyzed by a Kruskal-Wallis test. Then, selected combinations were compared by using the multcompare function. These analyses showed that *C. versicolor* exhibited the highest ability to degrade the main components of the lignocellulosic biomass (cellulose, hemicellulose and lignin) for all formulations as presented in Table 3. In particular, this fungus grown on F8 and F9 formulations showed the best degradations of cellulose. The highest lignin degradation corresponded to the F1 and F9 formulations again for *C. versicolor*. In the case of hemicellulose, *C. versicolor* exhibited the highest degradation when grown on F1, F9 and F12 media. An ANOVA was performed between F1

Table 2. Maximum lignocellulolytic enzyme activities of three white-rot fungi species for different carbon/nitrogen ratios and two levels of copper sulfate (II).

Enzyme activity	ENG	EXG	ENX	BG	LAC	MnP
P. ostreatus						
Maximum activity (U/g ss)	47.32	91.34	80.88	47.32	90.42	9.57
Day of max. activity	46	39	21	46	28	28
Medium formulation [a]	F3	F3	F6	F3	F3	F5
C. versicolor						
Maximum activity (U/g ss)	78.75	74.50	124.18	71.05	106.76	7.36
Day of max. activity	11	7	21	32	28	28
Medium formulation	F9	F5	F9	F5	F7	F3
L. edodes						
Maximum activity(U/g ss)	66.85	99.29	107.53	84.01	47.25	6.92
Day of max. activity	11	32	21	14	18	25
Medium formulation	F3	F5	F10	F2	F11	F4

[a]According to the formulations presented in Table 1. ENG, Endoglucanase activity; EXG, exoglucanase activity; ENX , endoxylanase activity; BG, β-glucosidase activity; LAC, laccase activity; MnP, manganese peroxidase activity; U, enzyme unit; g ss, gram of solid substrate.

and F9 formulations for *C. versicolor*, but the results indicated that there are no significant differences. Therefore, the combination *C. versicolor* – F9 medium was selected for further modeling considering that it presented high degradation values for these three biopolymers. In fact, this combination showed the highest average of the three mean degradation values.

Mathematical modeling

For the fungus/formulation combination exhibiting the highest average degradation of the main components of the lignocellulosic wastes used (*C. versicolor* – F9 medium), a non-structured non-segregated deterministic mathematical model was proposed in order to describe the behavior of the SSF process, particularly, the fungal growth, substrate degradation and lignocelullolytic enzyme production. In addition, the behavior of the reducing sugars (mostly glucose, cellobiose and xylose) was also included in the model considering that these sugars are intermediary products released and consumed during the utilization of the lignocellulosic biomass.

The proposed model is composed of 11 differential equations that are shown in Table 4. The values of the model parameters are as follows: μ_m = 6.7416 day^{-1}; C_{bm} = 358.0071 mg/g ss; n = 0.0279; q_p = 0.2636 mg×mg^{-1}×day^{-1}; k_L = 8.238x10^{-3} mg×g ss×day^{-1}×U^{-2}; k_{lac} = 1.341x10^{-3} U×g ss×mg^{-1}×mg^{-1}; k_{MnP} = 1.035x10^{-4} U×g ss×mg^{-1}×mg^{-1}; μ_{LAC} = 0 U×mg^{-1}×day^{-1}; μ_{MnP} = 5.659x10^{-4} U×mg^{-1}×day^{-1}; k_{HM} = 0.0245 mg×day^{-1}×U^{-1}; k_{ENX} = 2.353x10^{-3} U×g ss×mg^{-1}×mg^{-1}; μ_{ENX} = 0.2563 U×mg^{-1}×day^{-1}; k_C = 0.0101 mg×g ss×day^{-1}×U^{-2}; k_{ENG} = 2.511x10^{-3} U×g ss×mg^{-1}×mg^{-1}; μ_{ENG} = 0,08 U×mg^{-1}×day^{-1}; k_{EXG} = 5.064x10^{-4} U×g ss×mg^{-1}×mg^{-1}; μ_{EXG} = 0.0810 U×mg^{-1}×day^{-1}; k_{BG} = 0.0742 U ×mg^{-1}; μ_{BG} = 0.0256 U×mg^{-1}×day^{-1}. For *C. versicolor* grown on F9 medium, the following initial conditions were established: C_{b0} = 25.2333 mg/g ss; C_{AR0} = 12.8 mg/g ss; C_{L0} = 205.45 mg/g ss; C_{lac0} = 1.7374 U/g ss; C_{MnP0} = 0.5335 U/g ss; C_{HM0} = 225.36 mg/g ss; C_{ENX0} = 1 U/g ss; C_{C0} = 490 mg/g ss; C_{ENG0} = 1.888 U/g ss; C_{EXG0} = 0.4793 U/g ss; C_{BG0} = 3,999 U/g ss. The names of the 11 variables are indicated in the last column of Table 4.

The Equation 1 proposed for cell biomass description corresponded to a logistic model modified by Mitchell et al. (1999a). To describe the variation of reducing sugars, the Equation 2 including a constant production factor that affects growth rate was proposed; in this case, the change of these sugars had a better fit to the growth rate than to the cell biomass itself. The Equations 3, 6, and 8 for consumption of lignin, hemicellulose, and cellulose, respectively, were expressed as a function of the specific activities of the enzymes responsible for degradation of the corresponding substrates. The changes of cellulolytic (ENG and EXG), xylanolytic (ENX) and ligninolytic (LAC and MnP) specific enzyme activities (the equivalent of the concentration for enzymes) were considered to be dependent on the concentration of the corresponding substrate and on growth rate with an inhibition factor. This factor is related to the concentration of reducing sugars for the two cellulases and the xylanase as indicated in Equations 9, 10, and 7. In the case of ligninases, the inhibition factor was related to the lignin concentration in the medium as can be observed in Equations 4 and 5. Some authors (Tengerdy and Szakacs, 2003) state that the inhibition

Table 3. Degradation percentages of cellulose, hemicellulose, and lignin during the SSF using three white-rot fungi for different media formulations.

Formulation[a]	Cellulose			Hemicellulose			Lignin		
	C. versicolor	*P. ostreatus*	*L. edodes*	*C. versicolor*	*P. ostreatus*	*L. edodes*	*C. versicolor*	*P. ostreatus*	*L. edodes*
F1	37.83	29.49	27.83	37.86	35.65	34.45	65.22	46.01	50.39
F2	38.17	25.55	30.90	29.94	29.28	27.91	53.31	41.97	41.18
F3	39.18	29.84	29.79	35.92	32.68	33.96	58.96	41.60	45.55
F4	39.15	32.27	26.04	28.77	25.74	27.88	60.44	42.64	46.69
F5	42.88	30.28	28.49	29.41	25.42	26.55	57.67	40.68	44.55
F6	40.67	32.92	23.27	32.47	29.11	30.56	58.18	41.04	44.95
F7	37.35	31.13	27.04	33.02	32.90	31.33	55.93	44.94	43.21
F8	43.64	29.55	30.79	32.88	28.80	30.11	54.47	42.88	42.08
F9	43.21	29.68	28.26	36.54	26.50	29.54	63.69	44.93	49.21
F10	38.50	26.87	26.59	28.12	25.15	27.12	53.28	37.58	41.16
F11	34.71	26.89	24.82	32.32	26.88	30.28	53.94	38.05	41.67
F12	37.32	28.76	27.93	37.53	27.39	29.39	47.40	33.44	36.62

[a]According to the formulations presented in Table 1.

of these enzymes could be caused by intermediary compounds formed during the fermentation or, probably, by high lignin concentrations. Although there is still no certainty of the causes of this inhibition, the experimental data obtained in this work suggest a possible inhibition of ligninases; as no intermediary compounds were determined during the SSF, a substrate inhibition relationship was proposed, in this case, by the lignin. Finally, for description of BG production, Equation 11 was formulated as a dependence on the growth rate with an inhibition term affected by the reducing sugars concentration.

The follow-up of cell biomass was accomplished by determining the chitin contained in the cell wall of fungi through the quantification of NAGA at different times during the cultivation process. From parallel experimental runs under conditions of submerged fermentation, the NAGA content in the dry mycelium of *P. ostreatus*, *C. versicolor*, and *L. edodes* were determined. Thus, the NAGA content for these fungi were 15.83, 14.15, and 10.08 wt.%, respectively. These percentages were used to calculate the amount of cell biomass attached to the solid media. The behavior of the cell biomass during the fermentation is depicted in Figure 3 along with the time profile of the reducing sugars. As can be seen, the data fit was quite adequate. It should be noted that the model appropriately described the appearance/disappearance of the reducing sugars as key intermediary substances playing a crucial role during this type of SSF. The data fitting for lignin degradation and production of the corresponding ligninolytic enzymes (LAC and MnP) was satisfactory as well (Figure 4). The model was able to capture the decrease in the MnP activity after 20 h of cultivation. Finally, the data fitting was quite appropriate to describe the behavior of the hydrolytic enzymes released during the SSF using *C. versicolor* as can be observed in Figure 5 for hemicellulose consumption and ENX production, and in Figure 6 for cellulose consumption and production of cellulases measured (ENG, EXG, and BG). The suitability of the model was proven through 1-tailed *F*-test comparing the variance of the model residuals (deviation of the values calculated by the model related to the experimental data) and the variance of the experimental series for each variable considered in the model. The model was tested for all the 12 media formulations on which *C. versicolor* grew. This fact enabled to provide enough confidence about the validity of the representation of the experimental data by the model proposed.

DISCUSSION

Effect of C/N ratio and cupric sulfate on degradation of lignocellulosic wastes and enzyme production

The results presented in the present work regarding the C/N ratios used for formulation of the 12 solid media indicate that there exists no apparent direct

Table 4. Mathematical model describing the production of fungal biomass and lignocellulolytic enzymes, and consumption of lignocellulosic matrix.

Equation	Number	Description	Parameters and variables
$\frac{dC_b}{dt} = \mu_m . C_b \left(1 - \left(\frac{C_b}{C_{bm}} \right)^n \right)$	1	Biomass	μ_m: Specific growth rate (day^{-1}) C_{bm}: Maximum biomass concentration (mg/g ss) n: $n < 1$ The organism is relatively sensitive to the auto-inhibition and it occurs for very low values of C_b $n = 1$ Logistic equation $n > 1$ The organism is relatively resistant to the auto-inhibition and it occurs only when $C_b \approx C_{bm}$
$\frac{dC_{AR}}{dt} = q_P . \mu_m . \frac{dC_b}{dt} \left[1 - (n+1) \left(\frac{C_b}{C_{bm}} \right)^n \right]$	2	Reducing sugars	q_p: Production coefficient for reducing sugars ($mg \times mg^{-1} \times day^{-1}$) C_{AR}: Reducing sugars concentration ($mg \times g\ ss^{-1}$)
$\frac{dC_L}{dt} = -k_L . C_{LAC} . C_{MnP}$	3	Lignin	k_L: Lignin degradation coefficient ($mg \times g\ ss \times day^{-1} \times U^{-2}$) k_{LAC}: Laccase production coefficient ($U \times g\ ss \times mg^{-1} \times mg^{-1}$) k_{MnP}: Mn peroxidase production coeff. ($U \times g\ ss \times mg^{-1} \times mg^{-1}$)
$\frac{dC_{LAC}}{dt} = k_{LAC} . \frac{dC_b}{dt} . C_L - \mu_{LAC} . C_L$	4	Laccase	μ_{LAC}: Inhibition coefficient for laccase ($U \times mg^{-1} \times day^{-1}$) μ_{MnP}: Inhibition coefficient for Mn peroxidase ($U \times mg^{-1} \times day^{-1}$) C_L: Lignin concentration ($mg \times g\ ss^{-1}$) C_{LAC}: Laccase activity ($U \times g\ ss^{-1}$)
$\frac{dC_{MnP}}{dt} = k_{MnP} . \frac{dC_b}{dt} . C_L - \mu_{MnP} . C_L$	5	Manganese peroxidase (MnP)	C_{MnP}: Manganese peroxidase activity ($U \times g\ ss^{-1}$)
$\frac{dC_{HM}}{dt} = -k_{HM} . C_{ENX}$	6	Hemicellulose	k_{HM}: Hemicellulose consumption coefficient ($mg \times day^{-1} \times U^{-1}$) C_{HM}: Hemicellulose concentration ($mg \times g\ ss^{-1}$)
$\frac{dC_{ENX}}{dt} = k_{ENX} . C_{HM} . \frac{dC_b}{dt} - \mu_{ENX} . C_{AR}$	7	Endoxylanase	k_{ENX}: Endoxylanase production coefficient ($U \times g\ ss \times mg^{-1} \times mg^{-1}$) μ_{ENX} : Inhibition coefficient for endoxylanase ($U \times mg^{-1} \times day^{-1}$) C_{ENX}: Endoxylanase activity ($U \times g\ ss^{-1}$)
$\frac{dC_C}{dt} = -k_C . C_{ENG} . C_{EXG}$	8	Cellulose	k_C: Cellulose consumption coefficient ($mg \times g\ ss \times day^{-1} \times U^{-2}$) C_C: Cellulose concentration ($mg \times g\ ss^{-1}$)
$\frac{dC_{ENG}}{dt} = k_{ENG} . \frac{dC_b}{dt} . C_C - \mu_{ENG} . C_{AR}$	9	Endoglucanase	k_{ENG}: Endoglucanase production coeff. ($U \times g\ ss \times mg^{-1} \times mg^{-1}$) μ_{ENG} : Inhibition coefficient for endoglucanase ($U \times mg^{-1} \times day^{-1}$) C_{ENG}: Endoglucanase activity ($U \times g\ ss^{-1}$)
$\frac{dC_{EXG}}{dt} = k_{EXG} . \frac{dC_b}{dt} . C_C - \mu_{EXG} . C_{AR}$	10	Exoglucanase	k_{EXG}: Exoglucanase production coeff. ($U \times g\ ss \times mg^{-1} \times mg^{-1}$) μ_{EXG} : Inhibition coefficient for exoglucanase ($U \times mg^{-1} \times day^{-1}$) C_{EXG}: Exoglucanase activity ($U \times g\ ss^{-1}$)
$\frac{dC_{BG}}{dt} = k_{BG} . \frac{dC_b}{dt} - \mu_{BG} . C_{AR}$	11	β-glucosidase	k_{BG}: β-glucosidase production coeff. ($U \times mg^{-1}$) μ_{BG} : Inhibition coefficient for β-glucosidase ($U \times mg^{-1} \times day^{-1}$) C_{BG}: β-glucosidase activity ($U \times g\ ss^{-1}$)

Remarks: g ss – gram of solid substrate, U – unit of enzyme activity.

effect of this parameter on the variation of the six enzyme activities. This could be explained taking into account that the solid materials are not changed during the media formulation and that the levels of the C/N ratio evaluated (from 50 to 140) could not be different enough to cause significant variation in the enzyme activities measured. In this work, the data of ENG and EXG obtained were much lower than those ones obtained by other researchers like Kachlishvili et al. (2006) with different C/N ratios and for *L. edodes* and two *Pleurotus* species different to *P. ostreatus*. However, the EXG, BG, LAC, and MnP titers were higher related to those ones obtained with different C/N ratios for white-rot basidiomycetes reported by other authors (Baldrian and Gabriel, 2002; Reddy et al., 2003). Nevertheless, the enzyme titers can vary according to the efficiency of the method used to extract the enzymes from the solid substrate. In fact, the methodology employed could not necessarily ensure the extraction of all the enzymes absorbed to the substrate.

The presence of heavy metals affects the growth of

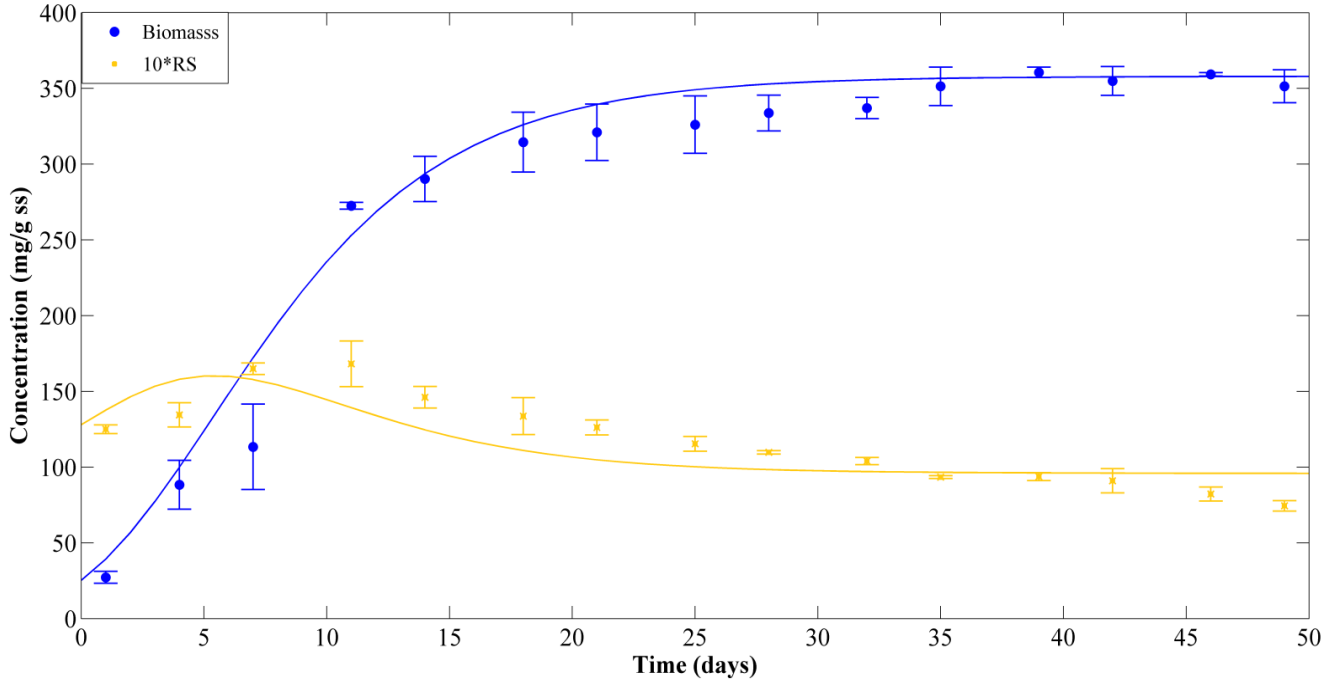

Figure 3. Time profile of cell biomass and reducing sugars for *C. versicolor* grown on the solid medium F9 based on oak sawdust, coconut husks, coffee husks, corn bran, and soybean oil under SSF conditions. The continuous lines were calculated by the model proposed. The values for reducing sugars are multiplied by 10.

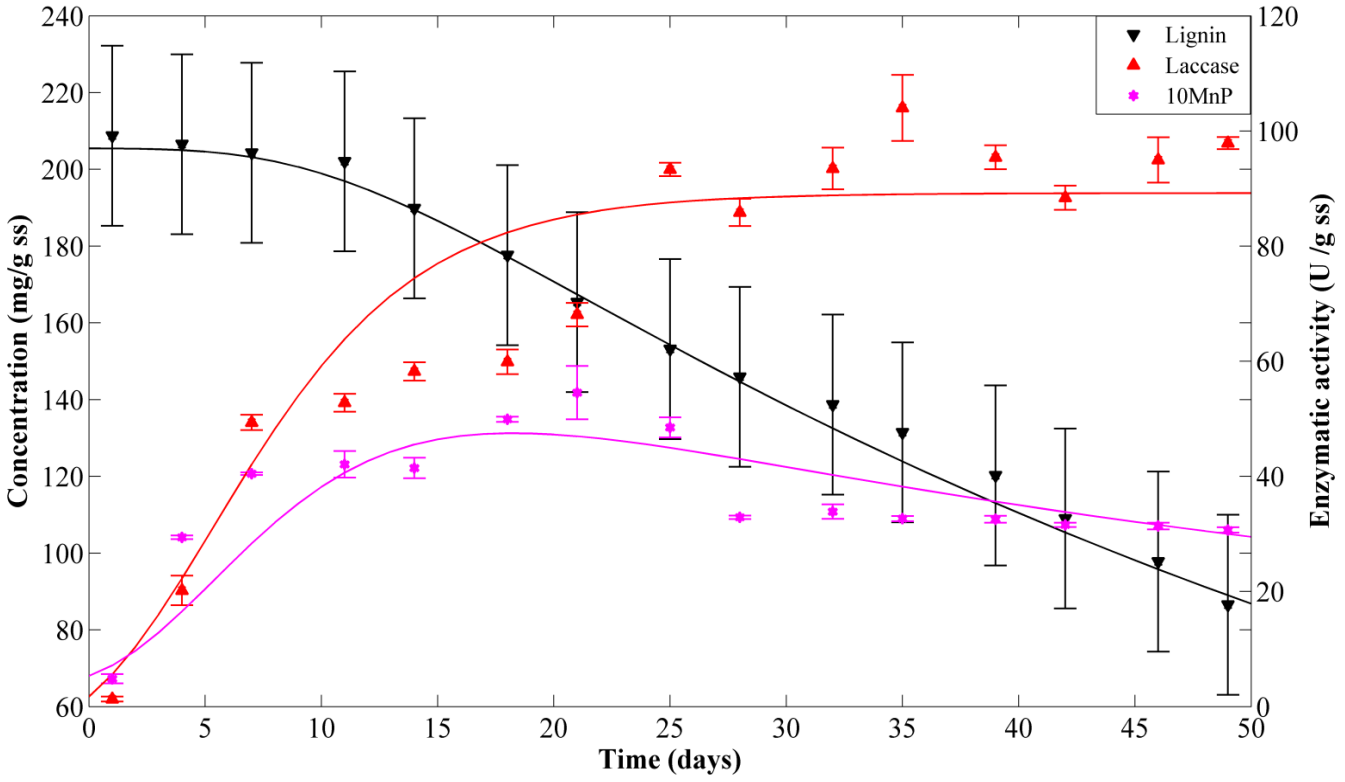

Figure 4. Time profile of lignin, laccase (LAC), and manganese peroxidase (MnP) for *C. versicolor* grown on the solid medium F9 based on oak sawdust, coconut husks, coffee husks, corn bran, and soybean oil under SSF conditions. The continuous lines were calculated by the model proposed. The values for MnP are multiplied by 10.

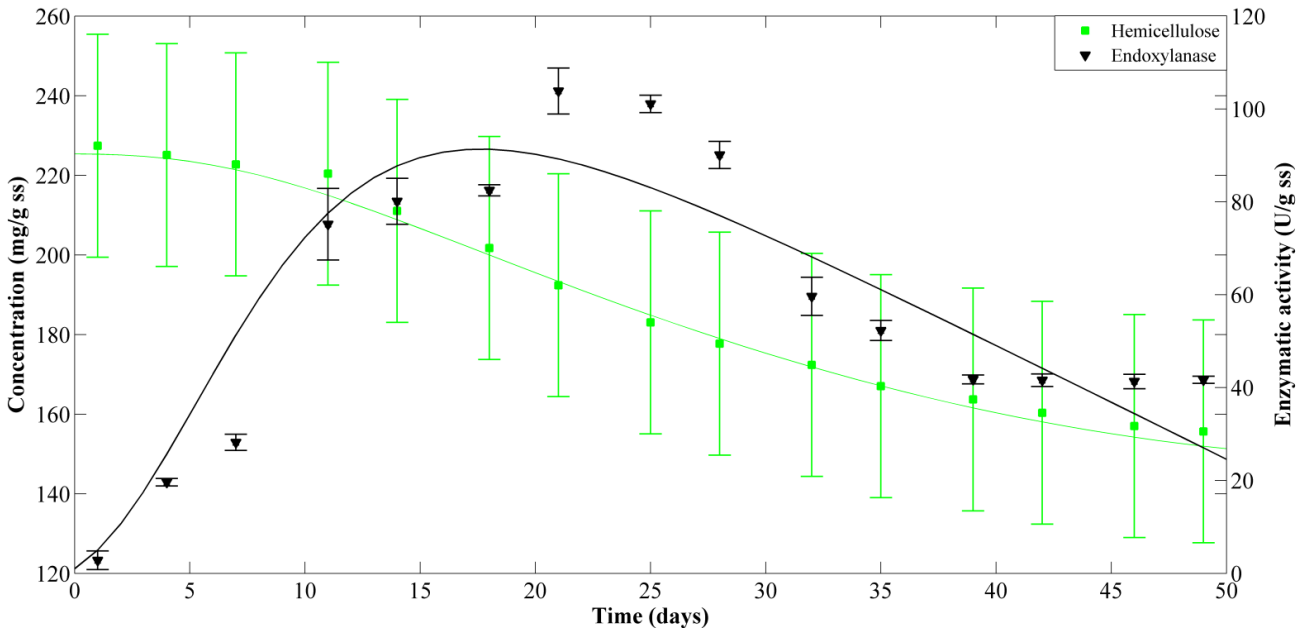

Figure 5. Time profile of hemicellulose and endoxylanase (ENX) for *C. versicolor* grown on the solid medium F9 based on oak sawdust, coconut husks, coffee husks, corn bran, and soybean oil under SSF conditions. The continuous lines were calculated by the model proposed.

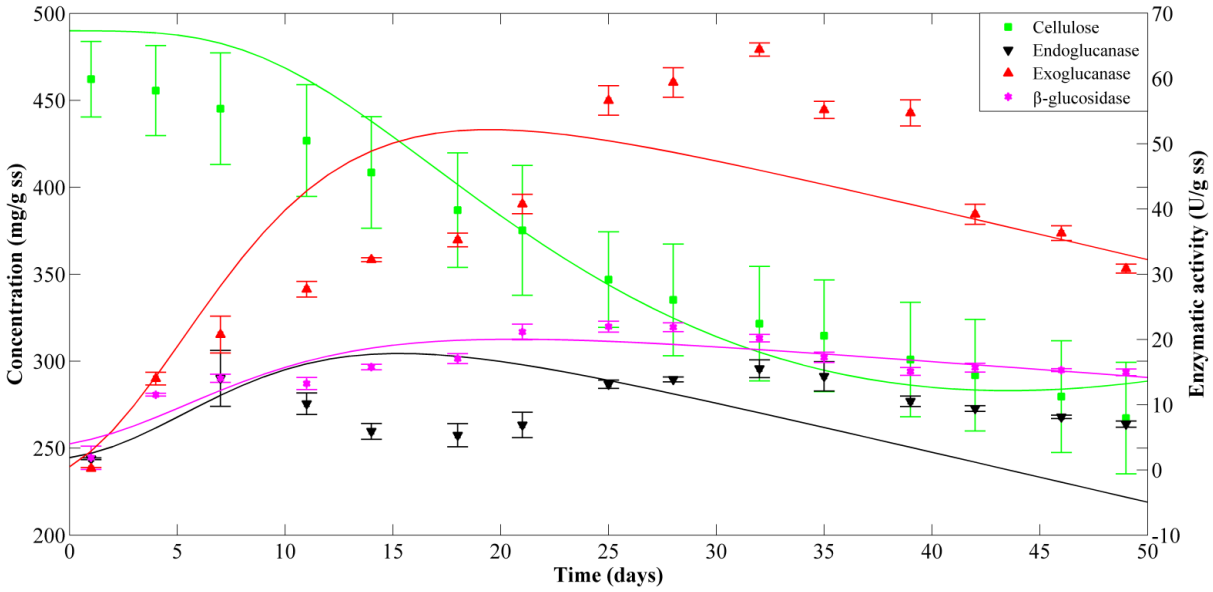

Figure 6. Time profile of cellulose, endoglucanase (ENG), exoglucanase (EXG), and β-glucosidase (BG) for *C. versicolor* grown on the solid medium F9 based on oak sawdust, coconut husks, coffee husks, corn bran, and soybean oil under SSF conditions. The continuous lines were calculated by the model proposed.

white-rot fungi. This effect can cause the decrease of fungal growth rate, which is sometimes accompanied with a prolonged lag phase. Thus, copper inhibited the growth of *Ganoderma lucidum* at concentrations less than 1 mM, while 150 ppm copper decreased the growth rate of *Phanerochaete chrysosporium* (Baldrian, 2003), but concentrations used in this work did not generate apparent inhibition on the growth of the three organisms analyzed.

Extracellular cellulolytic and ligninolytic enzymes are

regulated by heavy metals at the transcription level as well as during their catalytic action (Baldrian, 2003). The presence of heavy metals can also interfere with the carbon and energy supplying system of cellulases and hemicellulases. Cellulase of *P. chrysosporium* in liquid media was inhibited in the presence of 50-150 ppm cadmium, copper, lead, manganese, nickel and cobalt. Mercury, iron, and copper strongly inhibited the activity of β-glucosidase from *Trametes gibbosa* (Baldrian, 2003); but, in the range assayed in this work, copper had no influence on the production of cellulolytic and xylanolytic enzymes by the three fungi evaluated.

In the case of ligninolytic enzymes, several researchers have studied the effect of $CuSO_4$ addition as an inductor of laccase and peroxidases (Levin et al., 2002; Niladevi and Prema, 2008). Copper has been reported to be a strong laccase inducer in several species, among them *Ganoderma applanatum*, *Peniophora* sp., *Pycnoporus sanguineus*, and *Coriolus versicolor* f. *antarcticus* (Fonseca et al., 2010). It is known that copper induces both laccase transcription and activity (Collins and Dobson, 1997), and the increase in activity is proportional to the amount of copper added. Laccase production for all the three fungal species and 12 formulations could have been stimulated by $CuSO_4$ addition since LAC activities reached values higher than those ones reported in other works. For instance, in a previous report (Montoya et al., 2012), LAC activities of the white-rot fungus *Grifola frondosa* were not higher than 15 U/g ss (solid substrate) while activities as high as 90.42 U/g ss for *P. ostreatus* and 106.76 U/g ss for *C. versicolor* at 28 days were achieved in the present work. These activities are comparable to those obtained by Gassara et al. (2010) for one of the most studied basidiomycetes, *P. chrysosporium*, in presence of $CuSO_4$ on different lignocellulosic materials. These authors did not achieve any LAC activity when inducers were not used. Rosale et al. (2007) reported LAC activities comparable to this work as well. These authors showed an increase in LAC activity for *Trametes hirsuta* grown on ground orange peelings due to the addition of 1 mM cupric sulfate. Therefore, the presence of copper in cultivation media plays a crucial role for their induction and production. On the other hand, MnP activities did not show any considerable variation among the species and formulations. The activities obtained were comparable to those ones previously reported for *G. frondosa* (Montoya et al., 2012) and *P. chrysosporium* (Gassara et al. (2010) grown on lignocellulosic wastes.

Time course of enzyme production during SSF

The time course of the biosynthesis of the lignocellulolytic enzymes studied in this work showed interesting patterns as presented in the results section. Many basidiomycetes have the ability to simultaneously synthesize hydrolytic and oxidative enzymes, which are needed to degrade the substrates contained in the lignocellulosic complex. These enzymes are extracellular and inducible. In particular, the white-rot fungi show higher titers for ligninases than for cellulases. This implies an advantage for these fungi since they should start the lignin degradation in order to access the cellulose. In fact, the higher ability to degrade lignin by *C. versicolor* (Table 3) allowed a faster access to the cellulose leading to an enhanced cellulose consumption and, consequently, to an improved utilization of the carbon source.

The enzyme activities measured in this study presented decreases at different times during the SSF process. This fact could be explained by several issues like the efficiency of the extraction method employed; changes in the composition of the substrate during the fermentation since new compounds inhibiting the enzymes can be formed; repression exerted by the products generated during the enzymatic reactions; and synthesis of several isoenzymatic forms of the same enzymes at different times under different substrate conditions leading to different activities. This reduction in the enzyme activities has also been observed by other authors who have cited some of the above-mentioned causes for such decline (Mata et al., 2005).

Mathematical modeling

The SSF is a complex process that is strongly influenced by the cultivation conditions. The mathematical description of such process is very difficult considering several issues related to the determination of cell biomass (that is attached to the solid matrix), complex consumption or degradation of substrates, heterogeneity of the solid materials employed, and the impossibility to determine all the intermediary compounds generated from the biochemical reactions occurring during the different fermentation phases. For these reasons, the model proposed in this work should be considered as an attempt to describe the complex behavior of white-rot fungi grown on residual lignocellulosic materials. In fact, the modeling of the lignocellulolytic enzyme production by SSF has not been disclosed in the available literature for the case of macromycetes. The models proposed by other authors (Mitchell et al., 1999b; Mitchell et al., 2004; Viccini et al., 2001) have emphasized the fungal growth rate for micromycetes, and the biosynthesis of simple metabolites. From those works, the logistic equation was successfully applied in this paper to predict the behavior of the fungal biomass. However, the degradation of the fiber components (cellulose, hemicellulose, and lignin) and the synthesis of lignocellulolytic enzymes required mathematical expressions not reported before in order to fit the experimental data obtained as was done in the present work.

Although there exists an important amount of previous

works dealing with the production of lignocellulolytic enzymes by different white-rot fungi, degradation of lignocellulosic materials, and approximations to the determination of the intermediary compounds formed during SSF processes, a greater insight is required in order to generate the corresponding kinetic curves for growth rate, nutrient consumption, biopolymer degradation, and lignocellulolytic enzyme production. Undoubtedly, these kinetic relationships formulated within a comprehensive mathematical model are crucial to conceptually design SSF processes for enzyme or biomass production as well as for their scale-up and operation. As far as we know, this is the first attempt to model these complex processes in the case of *C. versicolor* using such heterogeneous materials as oak sawdust, coconut husks, coffee husks, corn bran, and soybean oil. The formulation of the model equations was made considering the biological sense of the phenomena studied. In fact, the expressions proposed acceptably described each one of the variables measured. For future works, it is necessary to take into account the key intermediary compounds formed during the SSF using white-rot fungi, or other enzymes not evaluated in this work along with their products. Furthermore, it is necessary to consider the intraparticle constraints to the heat and mass transfer during solid cultivation as studied by Mitchell et al. (2004). This will contribute to a deeper understanding of the complex phenomena occurring during the growth of macromycetes on lignocellulosic materials. The model proposed representing the kinetic relationships of the SSF process studied in this work is a powerful tool that could be used not only to provide valuable information on the process itself, but also to develop a commercial process for production of lignocellulolytic enzymes in the future. In addition, the model offers the possibility to reduce the number of experimental runs as well as to optimize the process.

Conclusions

In this work, three species of white-rot fungi were grown on 12 media formulations during 49 days of SSF varying their carbon/nitrogen ratios and their content of cupric sulfate. Some fungus/formulation combinations showed a promising performance regarding enzyme production. The fungus *C. versicolor* exhibited the highest ability to degrade the three main polymers of the lignocellulosic residual materials employed. The mathematical model proposed to describe the process studied could be used to provide valuable information on the process itself as well as to contribute to the development of a future commercial process for lignocellulolytic enzyme production.

Conflict of interest

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Assessment of acute toxicity profile of *Lasianthera africana* leaf extract in normal rats and its ameliorative effect in alloxan-induced diabetic rats

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The present study evaluated the acute toxicity effect of *Lasianthera africana* leaf extract on normal rats and effect of the extract on the hepatic and renal function indices in alloxan-induced diabetic rats. In the acute toxicity test, male Wister rats received orally four different single dose of the extract and were kept under observation for 14 days. The result indicates that LD_{50} of the extract is higher than 5,000 mg/kg body weight. For the second experiment, doses (50, 100 and 200 mg/kg body weight) of the leaf extract were administered orally to alloxan-induced diabetic rats for 28 days and the effect of the treatment on blood glucose, hepatic enzymes, serum proteins and renal function indices was evaluated. The result indicates progressive and significant ($p>0.05$) reduction in fasting blood glucose. The extract also exhibited significant ($p<0.05$) reduction in serum aspartate aminotransferase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP), and significant ($p<0.05$) increase in serum total proteins and globulin and marginal ($p>0.05$) reduction in serum creatinine and uric acid relative to the diabetic control group. The results suggest that the leaf extract is safe and may be beneficial in the management of diabetic mellitus.

Key words: *Lasianthera africana* leaf, acute toxicity, hepatotoxicity, renal function indices.

INTRODUCTION

The revelation of toxicity profile of plant products intended for use as a source of nutrients and non-nutrient health benefiting phytochemicals helps in the determination of the extent of its safety. This is because as important as it is for a product to be efficiently cheap and readily available, there is extreme need for such product to be safe for short and long term uses. The World Health Organization (WHO) has emphasized the fact that safety should be the over-riding criterion in the selection of herbal medicine for use in health care delivery (WHO, 1980). According to Lorke (1983), investigation of acute toxicity is the first step in the toxicological investigation of an unknown substance. The index of acute toxicity is the lethal dose 50 (LD_{50}). It is usually carried out on rats or mice by oral, intraperitoneal, intravenous or intramuscular routes of administration (Homburger, 1989). According to Homburger

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(1989), LD_{50} values should be reported in terms of the duration over which the animals were observed. The period may range from 24 h to 14 days or longer.

Lasianthera africana (*P. Beauv*) is one of the top six green leafy vegetables commonly consumed by the Efik and Ibibio ethnic groups in Nigeria (Williams et al., 2009). It belongs to the family Icacinaceae. The plant is called "editan" in Efik and Ibibio local dialects of Nigeria. It is a perennial, glabrous, shrub that reaches a height of 61 to 136 cm (Hutchinson and Dalziel, 1973). Among the Ibibios, four local varieties based on the leaf colour, taste and habitat are known. The varieties are the "afia", "akai", "idim", and "obubit" (Bassey et al., 2006). Traditionally, the leaves are utilized for both food and therapeutic purposes. The consumption of the leaf is high among rural communities where it is mostly found especially during the period that other leafy vegetables are out of season. It therefore plays a significant role in household food security. From prehistoric time, the plant has been exploited by traditional herbalists for the treatment of various ailments including typhoid fever, diarrhoea and candidiasis among other ailments (Andy et al., 2008). Ebana et al. (1996) reported that the leaf of *L. africana* is rich in chemical compounds of nutritional and therapeutic importance. Sofowora (1989) reported that the leaves are highly nutritive and are used in traditional concoctions for the treatment of ailments like constipation and general stomach aches. Aqueous extract of the leaf is usually taken orally or in enama form to treat indigestion, stomach discomfort and internal heat (Etukudo, 2003). According to Bassey et al. (2006), substantial levels of alkaloids, flavonoids, terpenes, anthraquiniones, phlobatannins, cardiac glycosides, saponins and tannins occur in the leaf. The presence of health promoting and protecting compounds such as alkaloids and flavonoids suggests that the leaf could be used to manage diabetes mellitus that is prevalence in our societies today.

Notwithstanding, the long term use of *L. africana* leaf and leaf products in soup preparation and traditional concoctions, there is dearth of scientific information on its toxicity profile. This study was therefore designed to assess the acute toxicity level of *L. africana* leaf extract in normal rats and the ameliorative effect of the extract in alloxan- induced diabetic rats.

MATERIALS AND METHODS

Twigs of white variety ("afia") of *L. africana* leaves were harvested from a garden at Aka Offot in Uyo, Akwa Ibom State, Nigeria and authenticated at the Taxonomy Unit of the Department of Botany and Ecological Sciences, University of Uyo, Nigeria. The leaves were destalked, washed in potable water, spread to surface air dry and cut (2 mm width). The cut leaf was blended with water (1:3 w/v) using Kenwood blender (Kenwood Ltd., Havant, UK). The blend was left for 2 h at room temperature (26±2°C) before filtering through 425 µm pore size sieve. The filtrate was stored at 4°C for subsequent use. A known volume of the filtrate was evaporated to dryness in a conventional oven (model P.P.22US, Genlab, England) and the weight of the residue was used to determine the concentration of the filtrate which was in turn used to determine the dose of administration of the extract (Achukwu et al., 2009; Ikewuchi and Ikewuchi, 2012).

Animal procurement and care

Three months old male albino rats weighing between 148 and 161 g obtained from the Animal Breeding Unit, Faculty of Basic Medical Sciences, University of Uyo, Akwa Ibom State, Nigeria were used for the experiment. Animals were housed in well ventilated stainless steel cages containing wood shavings for bedding. The animals were allowed to acclimatize for 7 days and maintained with standard grower's mash (UAC Vital Feed produced by Grand Cereals, Jos, Nigeria) and tap water *ad libitum* prior to experimentation. Animals were maintained under normal environmental temperature (26±2°C) with normal 12:12 h dark/light cycle. The room was cleaned and disinfected regularly. Soiled wood shavings were replaced weekly. The feed and water containers were also washed regularly. Each rat was marked for identification. The experiments were conducted in accordance with the internationally accepted principles for Laboratory Animal use and care as found in the US guidelines (NIH publication No. 85-23, revised in 1985).

Acute toxicity (LD_{50}) studies

The acute toxicity profile of the leaf extract was determined using the method described by Lorke (1983). Healthy male albino rats weighing between 148 and 160 g were randomly divided into five groups of five rats per group. The rats were deprived of food but allowed access to water *ad libitum* for 16 h prior to the administration of the leaf extract on day zero. The extract was administered orally at doses of 500, 1000, 3000 and 5,000 mg/kg body weight to determine the range within which toxicity lie. Rats in control group received no extract. All rats were monitored immediately after dosing and at intervals of 2 h for a total period of 12 h for signs of toxicity such as excitement, ataxia, alertness, nervousness and dullness. For the remaining of the 14 days study period, animals were monitored daily for mortality and signs of toxicity. Animal's body weight was measured prior to dosing on day 0 and on day 14. The number of survivors after the 14 day period was noted. On the last day, animals were sacrificed and the macroscopic evaluation of the liver and kidney was carried out to access the gross effect on the morphological features and appearance of the organs. The lethal dose (LD_{50}) was assessed on the bases of mortality (Angelis Pereira et al., 2005).

Inducement of diabetes

Alloxan monohydrate (Sigma-Aldrich Co., USA) was used to induce diabetes mellitus in normoglycaemic rats. Animals were allowed to fast for 16 h and were injected intraperitonially (i.p) with freshly prepared alloxan monohydrate in distilled water in a dose of 150 mg/kg body weight (Antai et al., 2010). Initial blood glucose was determined prior to inducement with alloxan and after 7 days of inducement to confirm diabetic state of the rats. Blood was collected from the tip of tail vein for glucose determination. Rats that showed fasting blood glucose levels above 230 mg/dl were selected for the study.

Experimental protocol

Twenty-five (25) alloxan-induced diabetic male albino rats were divided into five groups (groups 2-6) of five rats per group. Rats in group 1 (normal control) were given feed and drinking water only for

Table 1. Effect of oral administration of doses of *Lasianthera africana* leaf extract on body weight of normal rat after 14 days.

Dose levels (mg/kg body weight)	Initial weight (g) (day 0)	Final weight (g) (day 14)	Weight gain (%)
0.00	159.00^{b} ±3.61	173.00^{a} ±2.00	9.01
500	148.33^{b} ±2.08	164.00^{a} ±4.00	10.56
1.000	157.00^{b} ±2.00	170.33^{a} ±4.51	8.49
3.000	152.00^{b} ±2.65	164.00^{a} ±2.65	7.89
5.000	160.67^{b} ±3.51	173.00^{a} ±3.00	7.67

Values are means ± SD, n = 5. Means on the same row with different superscripts are significantly different at p<0.05.

28 days. Rats in group 2 (diabetic control) were also given feed and drinking water only for 28 days. Rats in group 3 were given feed, drinking water and treated with metformin (anti-diabetic drug) at a dose level of 100 mg/kg body weight daily (Tang et al., 2006) for 28 days. Rats in groups 4, 5 and 6 were treated daily with doses (50, 100 and 200 mg/kg body weight), respectively, of *L. africana* leaf extract for 28 days. They also had access to feed and drinking water *ad libitum* for the 28 days. The extract was given to the rats by oral administration using canula. The first day of administration was taken as day zero while the sacrifice day was on day 28. Fasting blood glucose was measured on the 0, 7, 14, 21 and 28 day of treatment. At the end of the treatment, animals were fasted overnight, but allowed access to water *ad libitum*. The rats were euthanized and ex-sanguinated under chloroform anaesthesia and their blood collected by jugular vein puncture (Wilson et al., 2001). The blood samples were dispensed into sterile plain tubes, allowed to stand for 3 h at room temperature (26°C) to ensure complete clotting and centrifuged at 3500 rpm for 10 min. The clear sera were aspirated off and stored at -20°C for biochemical evaluation.

Methods of analysis

Fasting blood glucose (FBG) was measured by single touch glucometer (One Touch Ultra 2 Blood Glucose Monitoring System, Lifescan Inc., Milpitas, USA). The weight of each rat was measured using Ohaus Electronic Weighing balance (Model CS 2000, USA). Biochemical indices were determined using standard ready-to-use reagent kits, (Randox Ltd., UK) following the manufacturer's instructions. The indices determined included aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total proteins, albumin, creatinine and uric acid.

Statistical analysis

The results of the studies were expressed as mean ± SD (standard deviation) of triplicate determinations. The data obtained were subjected to One-way Analysis of Variance (ANOVA) using SPSS version 18 statistical software package (SPSS, Inc., USA) to determine variation between treatments. *Post hoc* test was done using Tukeys test for multiple comparisons. Significant variation was accepted at $p < 0.05$.

RESULTS

Acute toxicity studies

L. africana leaf extract administered by oral route at doses up to 5,000 mg/kg body weight (single dose) did not produce any sign of toxicity or death in rats during 14 days of observation. Adverse clinical symptoms or toxicity signs like vomiting, diarrhoea, nervousness, ataxia and anorexia were not observed in the rats during the period of observation. Each treatment group exhibited significant (p<0.05) increase in body weight on the 14th day relative to the initial weight (Table 1). The percentage weight gained ranged from 7.67% for rats treated with 5,000 mg/kg to 10.56% for rats treated with 500 mg/kg body weight of the extract. Macroscopic examination of the livers and kidneys showed no discoloration, shrinkage, holes or other signs of abnormality.

Effect of aqueous extract on fasting blood glucose (FBG) in alloxan-induced diabetic rats

Table 2 shows the effect of oral administration of doses of *L. africana* leaf extract on the fasting blood glucose in alloxan-induced diabetic rats. Alloxan-induced diabetic rats had significantly (p<0.05) higher fasting blood glucose compared to that of normal control rats. The result indicated that the fasting blood glucose level of the normal control rats varied marginally while diabetic control rats showed progressive increase (p<0.05) in fasting blood glucose level throughout the 28 days period. The result of average weekly measurement of the fasting blood glucose revealed that daily treatment (oral administration) of diabetic rats with doses (50, 100 and 200 mg/kg body weight) of *L. africana* leaf extract resulted in significant (p<0.05) reduction in the fasting blood glucose. Compared with the glucose level before treatment (day 0), this hypoglycaemic effect of the leaf extract was sustained till the end of the study.

Effect of doses of *L. africana* leaf extract on serum hepatic function indices in alloxan-induced diabetic rats

Table 3 depicts the ameliorative effect of aqueous extract of *L. africana* leaf on AST, ALT, ALP and proteins in serum of alloxan-induced diabetic rats. The activities of

Table 2. Effect of doses of *Lasianthera africana* leaf extract on fasting blood glucose in alloxan-induced diabetic rats (mg/dl).

Treatment groups	Day 0	Day 7	Day 14	Day 21	Day 28
i	65.60^{a}±2.70	76.20^{b}±1.30	73.40^{b}±2.41	67.20^{a}±1.48	67.00^{a}±2.12
ii	235.60^{a}±3.91	309.40^{b}±3.05	317.80^{c}±4.76	346.00^{d}±3.16	377.80^{e}±6.14
iii	272.20^{a}±3.03	234.60^{b}±5.68	198.40^{c}±4.39	162.80^{d}±4.21	101.60^{e}±3.97
iv	281.00^{a}±4.30	262.60^{b}±5.18	231.60^{c}±3.05	155.20^{d}±4.76	108.20^{e}±5.26
V	242.40^{a}±2.28	215.20^{b}±3.19	138.40^{c}±5.32	120.80^{d}±3.70	87.40^{e}±4.72
Vi	290.60^{a}±2.70	216.40^{b}±5.32	219.40^{c}±4.83	146.40^{d}±4.34	111.20^{e}±3.89

Value are means ± SD, n = 5. Means across each row with different superscripts differ significantly ($p < 0.05$). i = normal control; ii = negative (diabetic) control; iii = diabetic + metformin (positive control); iv = diabetic + extract (50 mg); v = diabetic + extract (100 mg); vi = diabetic + extract (200 mg)

Table 3. Effect of doses of *Lasianthera africana* leaf extract on hepatic function indices in alloxan-induced diabetic rats.

Treatment groups	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Total proteins (g/dl)	Albumin (g/dl)	Globulin (g/dl)	Albumin/ globulin ratio
I	39.09±0.38	26.50±0.47	147.80±2.18	7.00±0.06	3.75±0.17	3.25±0.23	1.15
ii	65.60^{a}±0.58	51.64^{a}±0.74	306.98^{a}±7.54	5.31^{a}±0.28	2.11^{a}±0.21	3.20±0.19	0.66
iii	49.51^{ab}±0.58	38.14^{ab}±0.61	217.32^{ab}±3.35	6.69^{b}±0.16	3.83^{b}±0.33	2.86±0.48	1.34
iv	51.74^{abc}±0.29	36.87^{ab}±0.29	239.78^{abc}±3.57	6.21^{b}±0.30	3.35^{b}±0.31	2.86±0.17	1.17
v	42.75^{abc}±0.25	31.97^{abc}±0.40	206.74^{ab}±5.66	6.58^{b}±0.24	3.55^{b}±0.26	3.03±0.03	1.17
vi	45.39^{abc}±0.44	28.92^{abc}±0.50	151.60^{bc}±3.44	7.10^{b}±0.21	3.62^{b}±0.34	3.28±0.34	1.17

Value are means ± SD, n = 5. Means on the same column with different superscripts are significantly different at ($p < 0.05$). a = $p < 0.05$ (Test groups compared with group I). b = $p < 0.05$ (Compared with groups II). c = $p<0.05$ (Compared with groups III). i = normal control; ii = negative (diabetic) control; iii = diabetic + metformin (positive control); iv = diabetic + extract (50 mg); v = diabetic + extract (100 mg); vi = diabetic + extract (200 mg).

Table 4. Effect doses of *Lasianthera africana* leaf extract on renal function indices in alloxan-induced diabetic rats.

Treatment groups	Creatinine (mg/dl)	Uric acid (mg/dl)
I	0.51±0.02	3.81±1.17
ii	0.68^{a}±0.02	4.29±0.16
iii	0.53^{b}±0.07	3.76±0.29
iv	0.59±0.04	3.10±0.22
v	0.61±0.04	3.83±30.32
vi	0.57±0.02	3.73±0.25

Values are means ± SD, n = 5. Means on the same column with different superscripts are significantly different at $P < 0.05$. a=$p < 0.05$ (Test groups compared with I). b=$p < 0.05$ (Compared with groups II). i = normal control; ii = negative (diabetic) control; iii = diabetic + metformin (negative control); iv = diabetic + extract (50 mg); v = diabetic + extract (100 mg); vi = diabetic + extract (200 mg).

AST, ALT and ALP were significantly ($p<0.05$) elevated in the diabetic control rats relative to normal control rats. Treatment of alloxan-induced diabetic rats with doses (50, 100 and 200 mg/kg body weight) of *L. africana* leaf extract caused significant ($p<0.05$) reduction in AST by 21.13, 34.83 and 30.81%, in ALT by 28.60, 38.09 and 44.00% and in ALP by 21.89, 32.65 and 50.60%, respectively. The diabetic control rats showed marked proteins metabolic disturbances evident from their significant ($p < 0.05$) lower total protein (24.14%) and albumin (43.73%) compared to the normal control group. Treatment of diabetic rats with doses of the leaf extract for 28 days significantly ($p<0.05$) increased the serum total proteins by 16.99, 23.92 and 33.71% and albumin by 58.77, 68.25 and 81.04% for 50, 100 and 200 mg/kg body weight doses, respectively, compared to diabetic control rats. The albumin to globulin ratio of the diabetic control group was lower than those of the normal control and diabetic treated groups (Table 3).

Effect of doses of *L. africana* leaf extract on serum renal function indices in alloxan-induced diabetic rats

Table 4 shows the effect of oral administration of doses of *L. africana* leaf extract on serum creatinine and uric acid in alloxan-induced diabetic rats. The result shows that untreated diabetic rats (diabetic control) had significantly ($p < 0.05$) higher serum creatinine content and marginally

($p > 0.05$) higher uric acid content than the normal control group. Treatment of alloxan-induced diabetic rats with *L. africana* leaf extract (oral administration) for 28 days with daily doses of 50, 100 and 200 mg/kg body weight resulted in reductions ($p > 0.05$) of serum creatinine levels by 10.29, 13.24 and 16.18% and uric acid by 8.50, 18.85 and 10.78%, respectively, compared to the untreated diabetic rats.

DISCUSSION

The results of acute toxicity study indicate that *L. africana* leaf extract administered by oral route with the dose up to 5,000 mg/kg body weight did not produce any sign of toxicity or death in rats at the end of the 14 day observations. Weight loss which is a single and sensitive index of toxicity after exposure to toxic substances (Raza et al., 2002; Teo et al., 2002) were not observed in any of the treatment groups (Table 1). Based on the aforementioned observations, *L. africana* leaf extract can be considered non-toxic at the levels they were used. According to Kennedy et al. (1986), substances whose LD_{50} is higher than 5,000 mg/kg body weight by oral route can be considered practically nontoxic. In agreement with the results obtained in this study, Ikewuchi and Ikewuchi (2012) reported significant ($p<0.05$) increase in fasting blood glucose in alloxan-induced diabetic untreated rats. The reduction of fasting blood glucose in alloxan-induced diabetic rats as a result of daily oral administration of *L. africana* leaf extract (Table 2) signifies the hypoglycaemic potential of the extract. The most effective dose of the leaf extract was 100 mg/kg body weight as it produced 63.97% reduction in fasting blood glucose on day 28 compared to 61.50 and 61.73% reductions for groups that received daily dose of 50 and 200 mg/kg body weight, respectively. Similar low hypoglycaemic response at higher dose administration of some plant products have been reported by Kesari et al. (2005) and Mowla et al. (2009) for *Murraya Koenigii* and *Trigonella foenum-graecum* seed extract, respectively. The hypoglycaemic effect of the leaf extract compared favourably with the commercial anti-diabetic drug (metformin) that showed 62.68% fasting blood glucose reduction on the last day of the experiment. The active constituents responsible for hypoglycaemic effect in some plants have been identified to include diterpenes, flavonoids, alkaloids and steroidal glycosides among others (Akah et al., 2002; Li et al., 2004). These constitutents have been identified in *L. africana* leaf (Bassey et al., 2006) and may probably be responsible for the observed hypoglycaemic effect of the leaf extract. This result suggests that the extract of *L. africana* leaf can be used in the management of diabetes mellitus.

According to Hilaly et al. (2004), AST, ALT and ALP are considered markers for liver function. Elevation of these enzymes in serum serves as an indicator of hepatotoxicity (Elizabeth and Harris, 2005). AST and ALT are usually found in large quantities in the liver where they play an important role in the metabolism of amino acid (Whitehead et al., 1999). ALP on the other hand is predominantly found in the bile duct of the liver, and is considered as an indicator of biliary function, cholestasis and hepatic function (Whitehead et al., 1999; Elizabeth and Harris, 2005). The elevated levels of AST, ALT and ALP in the serum of diabetic control rats (Table 3) suggest hepatocellular damage which could cause their leakage from the hepatocytes into the blood circulation (Elizabeth and Harris, 2005). Stanely et al. (2000) noted that experimental diabetes induced by alloxan in rats cause tissue damage in the pancreas, liver, kidneys and heart, which can be reflected by an increase of AST and various hepatic enzymes such as ALT. The result of this study corroborate those of other authors who also reported increased AST, ALT and ALP in serum of alloxan-induced diabetic rats (Moram, 2001; Nwanjo, 2007; Akah et al., 2009; Ikewuchi and Ikewuchi, 2012). Similar decreases in AST, ALT and ALP in the serum of alloxan-induced diabetic rats following treatments with various plant extracts had been reported by Moram (2001), Nwanjo (2007), Akah et al. (2009) and Ikewuchi and Ikewuchi (2012). The result of this study indicates that *L. africana* leaf extract contained constituents that exerted hepatoprotective effect in alloxan-induced diabetic rats. The actual mechanism by which the leaf extract reduced elevated serum levels of AST, ALT and ALP in alloxan-induced rats remains unclear. Rawi et al. (1998) noted that the decrease of transaminase activities with treatments may be attributed to improved liver function with the return of gluconeogenesis towards its normal state. Determination of serum protein like albumin can act as a criterion for assessing synthetic capacity of the liver, since nearly all of them are synthesized in hepatocytes (Rasekh et al., 2008). Decreases in serum proteins as observed in diabetic control rats (Table 3) reflects chronic liver damage. Similar observation was reported by Akah et al. (2009). The common pattern seen following significant hepatocellular damage is a reduction in albumin accompanied by a relative increase in globulin which leads to reduce albumin to globulin ratio (Woodman, 1996). The implication of this result is that *L. africana* leaf extract has potentials to prevent hepatocellular damage in diabetic animals.

In the present study, creatinine and uric acid determinations were critical as markers of kidney function (Newman and Price, 1999). Creatinine is excreted out of the body entirely by the kidneys while uric acid is excreted to a large degree by the kidneys and to a smaller degree in the intestinal tract by microbial degradation (Sood, 2006). Elevated creatinine and uric acid levels in the serum of untreated diabetic rats signify impaired kidney function or disease (Sood, 2006). Similar higher levels of serum creatinine and uric acid in alloxan-induced diabetic rats had been reported by Akah et al. (2009) and Alarcon – Aguilar et al. (2005), respectively.

Reductions of serum creatinine and uric acid levels as a result of treatment with the extract indicate that the leaf extract exhibited potentials to preserve the renal integrity of the treated rats. The observed apparent kidney protection exhibited by the leaf extract may be associated with the inherent heterogeneous phytochemicals present naturally in the leaf. Reduction of uric acid in the treated rats suggest that therapeutic advantage can be taken of the leaf extract ability to reduce uric acid in hyperuricemia, a condition that can pre-dispose to gouty arthritis, intense inflammation of soft tissues on which urate crystals are deposited (Rock et al., 1986).

Conclusion

The results obtained herein indicate that *L. africana* leaf extract administered by oral route is safe. The study shows that the extract was hypoglycaemic and also revealed the hepato-renal protective potentials of the extract in alloxan-induced diabetic rats. Further biochemical investigations are proposed to elucidate the mechanism of action of this extract.

Conflict of interests

The authors did not declare any conflict of interest.

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Electrophoretic analysis of proteins from different date palm *(Phoenix dactylifera* L.) cultivars in Saudi Arabia

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Fifteen (15) samples of different date palm cultivars were collected from different locations in Al-Ahsa oasis in the eastern province of Saudi Arabia. Extracted proteins from these samples were analyzed by electrophoresis, and clustered according to the average linkage between groups hierarchical clustering method. The results reveal high degree of similarity based on Jaccard`s similarity method on the basis of presence and absence of bands, that ranged between 0.421 to 0.917, which was represented by phylogenetic dendrogram in six clusters. The closely related cultivars "Hel" and "Hat" in addition to "Khl" represent the sixth cluster, which is separated out of other cultivars with high degree of similarity that ranged between 0.636 to 0.714; it was confirmed by the first principal component with high loading (52.3%), and characterized by four bands (92, 100, 205 and 108 kda). These bands were mostly positioned close to each other in the scatter diagram. The second principal component with loading of 15.7%, which were represented by three bands 19, 25, and 37 kda, have been confirmed the first cluster of closely related cultivars "Shi" and "Shl", as well as the closely related cultivars "Mj" and "OmR" among the second cluster. It can be concluded that most of Al-Ahsa oasis date palm cultivars were from one genetic origin, however, each cultivar was grown from seed of locally known cultivar, and later was selected due to preferred fruit characteristics. More biochemical and molecular studies would be necessary to uncover the genetic relationships between area cultivars.

Key words: SDS-PAGE, bands, phylogenetic, cluster, dendrogram, similarity, principal components.

INTRODUCTION

Date palm (*Phoenix dactylifera* L., 2n=2x=36) is a monocotyledonous and dioeciously species belonging to Arecaceae family. It includes 225 genus and 2600 species (Corner, 1966), and widely cultivated in arid regions of the middle east and north Africa, (Hamza et al., 2011; Khierallah et al., 2011; El-Tarras et al., 2007; Elmeer et al., 2011). It is widely distributed in the Eastern Province of Kingdom of Saudi Arabia. There are more than 70 cultivars that have been grown there for ages (Asif et al., 1982; Al-Ghamdi and Al-Kahtani, 1993a; Al-Issa, 2009, 2013). Date palm can be propagated by seeds, which usually produce trees bearing inferior fruits. Offshoots are more preferred for conventional propagation because they produce true-to-type trees with

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Table 1. Cultivars names with their corresponding abbreviations.

Cultivar	Abbreviation
Mejnaz	Mj
Khalas	Khl
Shaishi	Shi
Shehil	Shl
Garrah	Grr
Berhi	Brh
Helali	Hel
Hatemi	Hat
Khonaizi	Khn
Asailah	Asi
Khosab	Khs
Tayyar	Tya
OmRohaim	OmR
Ruzaiz	Ruz
Owsaili	**Ows**

fruit quality identical to the mother tree (Asif et al., 1982; Al-Ghamdi and Al-Kahtani, 1993b, c; Khierallah et al., 2015). The high stability of protein profile makes protein electrophoresis a powerful tool in elucidating the origin and the evolution of cultivated plants (Ladizinsky and Hymowitz, 1979), as well as fast and less expensive alternate tool (Smykal et al., 2008). Therefore, protein pattern analysis by gel electrophoresis has been used in higher plants to study various problems in genetics (Mohammed et al., 2006; Dakhil et al., 2013; Koshroo et al., 2011), taxonomy (Barta et al., 2003), physiology (Stegemann et al*.,* 1987; Al-Helal, 1994), as it has been appreciated as a biochemical tool for studying the phylogentic relationships (Al-Yahyai and Al-Khanjari, 2008; Attaha, et al., 2013). The phylogenetic analysis based on protein patterns were used to study the genetic relatedness between and among cultivars (Abd El-Hady et al., 2010; Munshi and Osman, 2010; El Akkad, 2004; Haider et al., 2012; Khoshroo et al., 2011; Attaha et al., 2013). Protein patterns were used in identification of different date palm cultivars (Stegemann et al*.,* 1987; Munshi and Osman, 2010; Koshroo et al., 2011; Attaha et al., 2013; Koshroo et al., 2013; Al-Issa, 2013), as well as in different plants (Barta et al., 2003; El Akkad 2004; Abd El-Hady et al., 2010; Gad and Mohamed, 2012; Aejaz et al., 2014). So, it would be helpful in recognizing the relationships of different cultivars. Fifteen cultivars namely: Mejnaz, Khalas, Shaishi, Shehel, Garrah, Berhi, Helali, Hatemi, Khonaizy, Osailah, Khossab, Tayyar, Omm rhaim, Ruzaiz, and Awosaili were selected for this study, where Khonaizi and Khossab cultivars were known as Qatif origin cultivars, also Berhi cultivar was known as Iraqi origin cultivar. The main objective of this study was to enrich our knowledge about the biodiversity of Al Ahsa oasis date palm cultivars, however, it aimed to recognize inter-genetic relationships, phylogenetic revolution using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) technique.

MATERIALS AND METHODS

The samples of the 15 cultivars were collected from juvenile green leaflets of 30 years old and above of date palm cultivars trees. The samples transferred immediately to liquid nitrogen, then to deep freezer -20°C until the time of usage. The samples were cut into 2 × 2 mm before ground in mortar with liquid nitrogen prior to be subjected to protein electrophoretic analysis using the SDS-PAGE (Table 1).

Protein extraction

The samples were ground in a mortar by liquid nitrogen, then the proteins extracted with 0.7 ml extracting buffer (0. 5 M Tris/HCl buffer, pH 6.8 + 10% Glycerol v/v + 4% PVP w/v) according to Al-Helal (1994) with some modifications, by homogenizing 50 mg of ground leaflet samples by half volume stainless steel beads at 9/4 min., followed by 10/2 min by using bullet blender homogenizer, then incubated overnight at 4°C. The crude extract was vortexed by using VELP vortex mixer, then centrifuged at 12500 rpm for 10 min by using Eppendorf centrifuge 5424; the supernatant was moved to new tubes, while the debris was discarded.

Protein concentration and resuspending

According to Wessel and Fluegge (1984), with some modifications, to 150 µl of the supernatant, 600 µl methanol was added, vortexed, next 150 µl chloroform was added, vortexed, 450 µl deionized water was added, vortexed, centrifuged at 10000 rpm for 1 min, then top aqueous layer was removed, while protein was between layers, 600 µl methanol was added, vortexed, centrifuged at 10000 rpm for 1 min, then supernatant was removed without disturbing pellet; precipitated protein was incubated at room temperature for 15 min; the precipitated proteins was dissolved in 150 µl of extracting buffer, vortexed, boiled at 95°C for 4 min, then vortexed, centrifuged at 12500 rpm for 10 min, after that loaded in 15 µl.

Electrophoresis

Discontinuous vertical SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970) with some modifications, in 12.5% resolving gels [6.67 ml Acrylamide - bis acrylamide (30/2.6%) + 4 ml 1.5 M tris/HCl buffer pH 8.8 + 5.05 ml DW + 0.16 ml 10% SDS + freshly prepared 0.12 ml (10% amm. persulphate) + 0.008 ml TEMED] and stacking gels [0.95 ml Acrylamide - bis acrylamide (30/2.6%) + 1.25 ml 0.5 M tris/HCl buffer pH 6.8 + 2.72 ml DW + 0.05 ml (10% SDS) + freshly prepared 0.025 ml (10% amm. persulphate) + 0.005 ml TEMED]. The run buffer was prepared by dissolving 3.0 g Tris + 14.1 g Glycine + 1 gm SDS, in DW to 1 L, while the pH was adjusted to 8.3, and the electrophoresis was carried out by using (BioRad, Broka 0.75 mm) mini electrophoresis system with (BioRad PowerPac Basic) at 100 to 150 V.

Staining with coomassie and destaining

The gel was stained by coomassie brilliant blue R-250, and destained according to Meyer and Lambert (1965) with some modifications.

Table 2. Protein pattern bands represented by O = absent and I = present.

Mwt.	Mj[a]	Khl[b]	Shi[c]	Shl[d]	Grr[e]	Brh[f]	Hel[g]	Hat[h]	Khn[i]	Asi[j]	Khs[k]	Tyr[l]	OmR[m]	Ruz[n]	Ows[o]
270	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
260	O	O	O	O	O	O	I	I	O	I	I	I	I	O	O
239	O	O	O	O	O	I	O	O	O	O	O	O	O	O	O
205	O	I	O	O	O	O	I	I	O	O	O	O	O	O	I
165	O	O	O	O	O	O	O	O	O	O	O	O	O	O	I
150	O	O	O	O	I	I	I	I	O	I	O	O	O	O	O
145	I	O	O	O	O	O	O	O	O	O	O	O	O	I	I
108	O	I	O	O	O	I	O	I	O	O	O	O	O	O	O
100	O	I	O	O	O	O	I	I	O	O	O	O	O	O	O
92	O	I	O	O	O	O	O	I	O	O	O	O	O	O	O
84	I	I	I	O	O	O	O	I	O	O	O	O	I	I	I
75	I	O	O	O	O	I	I	I	O	O	O	O	I	O	O
60	I	I	O	O	I	I	O	O	O	O	I	O	I	I	O
56	I	I	I	I	I	I	I	O	O	O	I	O	I	I	I
50	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
4**6**	O	O	O	O	I	O	O	O	O	O	O	O	O	O	O
40	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
37	O	I	O	O	O	O	I	O	O	O	O	O	I	O	O
35	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
32	O	O	O	O	O	O	I	O	I	O	O	O	O	I	O
28	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
25	I	I	I	I	O	I	I	I	O	O	O	O	I	O	O
22	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
19	O	O	O	O	O	O	O	O	O	I	O	O	O	O	O
17	I	I	I	I	I	I	I	I	I	I	I	O	I	I	I
15	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
5	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Total	15	18	12	11	13	16	18	18	10	12	12	9	16	14	14

Staining with silver nitrate plus and destaining

Completely the gel was destained from coomassie brilliant blue R-250, then stained by silver nitrate according to BIO-RAD silver staining after Coomassie Brilliant Blue R-250 staining (Silver Stain Kit-161-0443).

Band scoring and analysis

Protein bands were scored, only unambiguous bandscoded for presence / absence (I/O). Quantitative evaluation of the protein bands was done by eye.

Protein profiling

Molecular weight of electrophoretic protein bands were calculated according to Weber and Obero (1969) method using standard protein marker with the following bands starting at the top with 250 K.D band followed by 150, 100, 75, 50, 37, 25, 20, 15 and 10 K.D band (Table 2).

Data analysis

The results obtained from protein patterns were analyzed statistically, while molecular weight of each protein band was determined (Table 2). Protein bands were scored depending on their presence (I) or absence (O). Jaccard`s similarity was determined and hierarchical clustering was constructed, principal components analysis PCA was done by using IBM SPSS Statistics for Windows software (2010).

RESULTS

The total protein extracts of the different cultivars of the date palm trees, which were collected from leaflets samples were subjected to SDS-PAGE analysis, then stained by Coomassie brilliant blue R-250, and re-stained by silver nitrate plus. Therefore, the faint bands which developed by Coomassie brilliant blue R-250 stain, became clearer when re-stained by silver nitrate plus stain. In general, the protein pattern of studied cultivars visibly looked slightly different, however, these differences were seen in the low protein content bands, as well as there is differences related to the intensity of protein bands. The proteins were found to be composed of a total of 27 bands (Figures 1 and 2, Table 2). Some

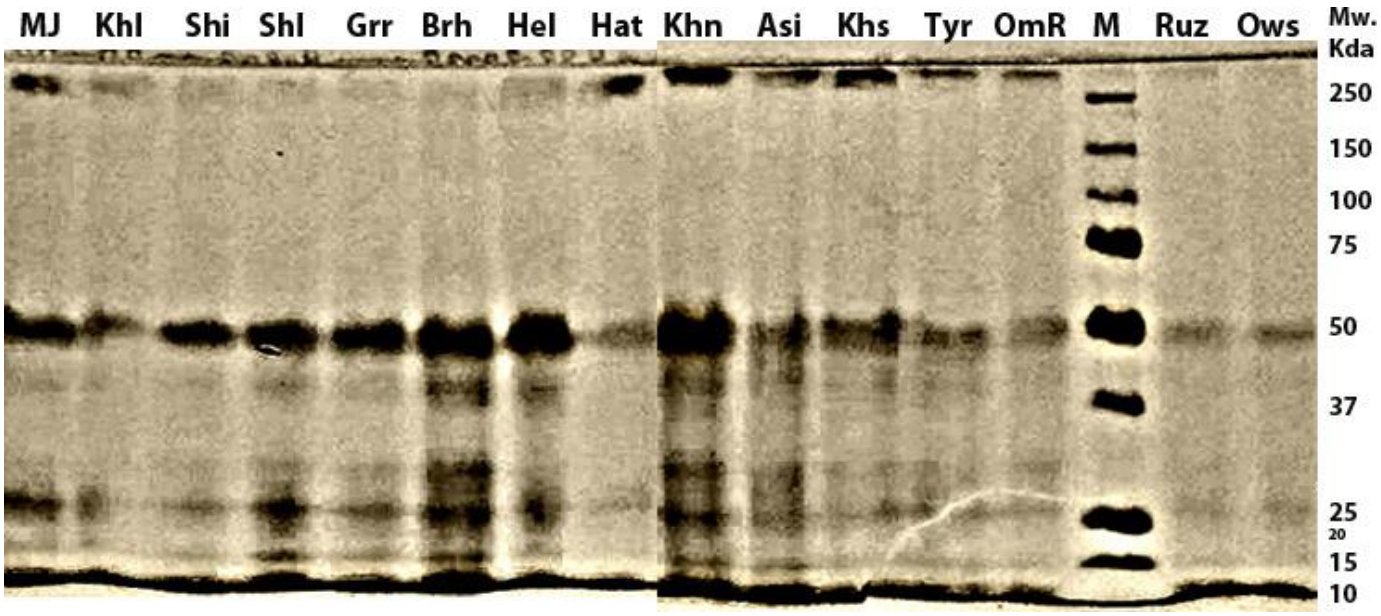

Figure 1. Electrophoretic proteins patterns of cultivars samples with protein standard marker, stained by comassie blue R-250.

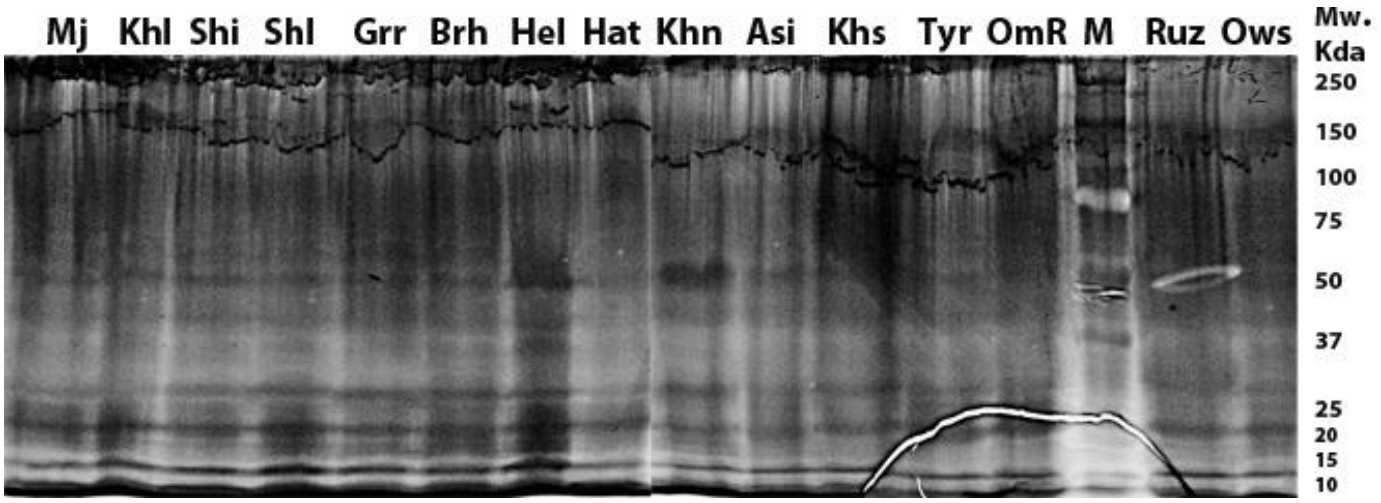

Figure 2. Electrophoretic proteins patterns of cultivar samples with protein standard marker, restained by silver nitrate.

bands were common between all cultivars at: 270, 50, 40, 35, 28, 22, 15 and 5 kda, while most bands number (18) occurred in "Khl", "Hel" and "Hat" cultivars, while the least number (9) occurred in "Tyr" cultivar. Protein bands varied in the intensity between studied cultivars; appeared higher in "Hel" cultivar, and lower in "Hat" cul-tivar. "Hel" and "Khn" cultivars seemed higher in intensity of band 50 kda than other cultivars, as well as "Khl" and "Hel" cultivars which seemed higher in intensity of band 22 kda than other cultivars bands (Figure 1 and 2, Table 2).

The genetic similarity based on Jaccard`s similarity method on the basis of presence and absence of bands (Table 2), ranged between 0.421 to 0.917. The highest similarity value of 0.917 based on Jaccard`s similarity method was observed between "Shi" and "Shl" cultivars, while the least similarity value of 0.421 was observed between "Khl" and "Tyr" cultivars. Protein banding pattern of "Brh" cultivar sample was at 239 kda, "Ows" cultivar sample was at 165 kda, "Grr" cultivar sample was at 46 kda, and "Asi" cultivar sample wat 19 kda showing allelic variations, but were resolved with low protein content (Table 3).

Phylogenetic analysis (Figure 3) showed six clusters; the first cluster composed of "Shi" and "Shl" cultivars,

Table 3. Data matrix of Jaccard`s Similarity analysis within different cultivars.

Molecular weight	Mj[a]	Khl[b]	Shi[c]	Shl[d]	Grr[e]	Brh[f]	Hel[g]	Hat[h]	Khn[i]	Asi[j]	Khs[k]	Tyr[l]	OmR[m]	Ruz[n]	Ows[o]
Mj[a]	1														
Khl[b]	0.650	1													
Shi[c]	0.800	0.667	1												
Shl[d]	0.733	0.611	0.917	1											
Grr[e]	0.647	0.550	0.667	.714	1										
Brh[f]	0.722	0.619	0.647	.688	0.706	1									
Hel[g]	0.571	0.636	0.579	.611	0.550	0.619	1								
Hat[h]	0.571	0.714	0.579	.526	0.476	0.619	0.714	1							
Khn[i]	0.563	0.474	0.692	.750	0.643	0.529	0.556	0.474	1						
Asi[j]	0.500	0.429	0.600	.643	0.667	0.556	0.579	0.579	0.692	1					
Khs[k]	0.750	0.550	0.667	.714	0.733	0.706	0.632	0.550	0.643	0.667	1				
Tyr[l]	0.500	0.421	0.615	.667	0.571	0.471	0.500	0.500	0.727	0.750	0.692	1			
OmR[m]	0.824	0.700	0.750	.688	0.611	0.684	0.700	0.619	0.529	0.556	0.813	0.563	1		
Ruz[n]	0.813	0.600	0.733	.667	0.688	0.579	0.524	0.455	0.714	0.529	0.688	0.533	0.667	1	
Ows[o]	0.706	0.600	0.733	.667	0.588	0.500	0.524	0.524	0.600	0.529	0.588	0.533	0.579	0.750	1

a, Mejnaz; b, Khalas; c, Shaishi; d, Shehel; e, Garrah; f, Berhi; g, Helali; h, Hatemi; i, Khonaizi; j, Asailah; k, Khosab; l, Tayyar; m, Omruhaim; n, Ruzaiz; o, Owsaili.

which were closely related, and distinguished by 11 common bands and degree of similarity (0.917); the second cluster composed of "Mj", "OmR" and "Khs" cultivars, were "Mj" and "OmR" were closely related, and distinguished by 14 common bands and degree of similarity of 0.824; the third cluster composed of "Ruz" and "Ows" cultivars, which were closely related, and distinguished by 12 common bands and degree of similarity of 0.750; the fourth cluster composed of "Grr" and "Brh" cultivars, which were closely related, and distinguished by 12 common bands and degree of similarity 0.706; the fifth cluster composed of "Asi", "Tyr" and "Khn" cultivars, were "Asi" and "Tyr" closely related, and distinguished by nine common bands and degree of similarity 0.750, the sixth cluster composed of "Hel", "Hat" and "Khl" cultivars, were "Hel" and "Hat" closely related, and distinguished by 15 common bands and degree of similarity 0.714 (Table 2).

Principal components analysis PCA was used to evaluate the results of electrophoretic patterns depending on presence (I) or absence (O) of electrophoretic bands (Table 2); the KMO and Bartlett`s tests for adequacy and sphericity result where 0.722 and 0.001, respectively (Table 4), the variance results were summarized in (Table 5), since the first component represent 52.3% of the total variations, the second component represent 15.7% of the total variations, hence the cumulative value represent 68% of the total variations, however the first component was represented by four variables, 92, 100, 205 and 108 kda, the second component was represented by three variables,19, 25, and 37 kda (Table 6); the variables were scattered on the 2-dimensional scatter gram, where both first and second principal components have been represented by their variables in two distinct groups (Figure 4).

DISCUSSION

Electrophoretic protein patterns technique reproduce considerable results in discrimination of differences among species and cultivars, however the date palm leaflets were wide used by researchers as a source of whole proteins for studies related to phylogenetic revolution or cultivars identification (Ahmed and Al Qaradhawi, 2009; Dakhil et al., 2013; Al-Issa, 2013; Khierallah et al., 2014). Validity of the results data was based on presence and absence of electrophoretic bands (Table 2), for principal components analysis (Andy, 2005), since the data have passed the tests of KMO and Bartlett for adequacy and sphericity, with 0.722 and 0.001, respectively. The study revealed eight bands in common between all cultivars at 270, 50, 40, 35, 28, 22, 15 and 5 kda. So, the high degree of similarity based on Jaccard's similarity method on the basis of presence and absence of bands, which ranged between 0.421 to 0.917 (Table 3), have been represented by phylogenetic dendrogram in the six clusters since each cluster includes a condition of closely related cultivars, "Shi" and "Shl", "Mj" and "OmR", "Ruz" and "Ows", "Grr" and "Brh", "Asi" and "Tyr", "Hel" and "Hat" with degrees of similarity 0.917, 0.824, 0.750, 0.706, 0.750 and 0.714, respectively. It could be supposed that each cluster were evolved from one common ancestor, as well as it could

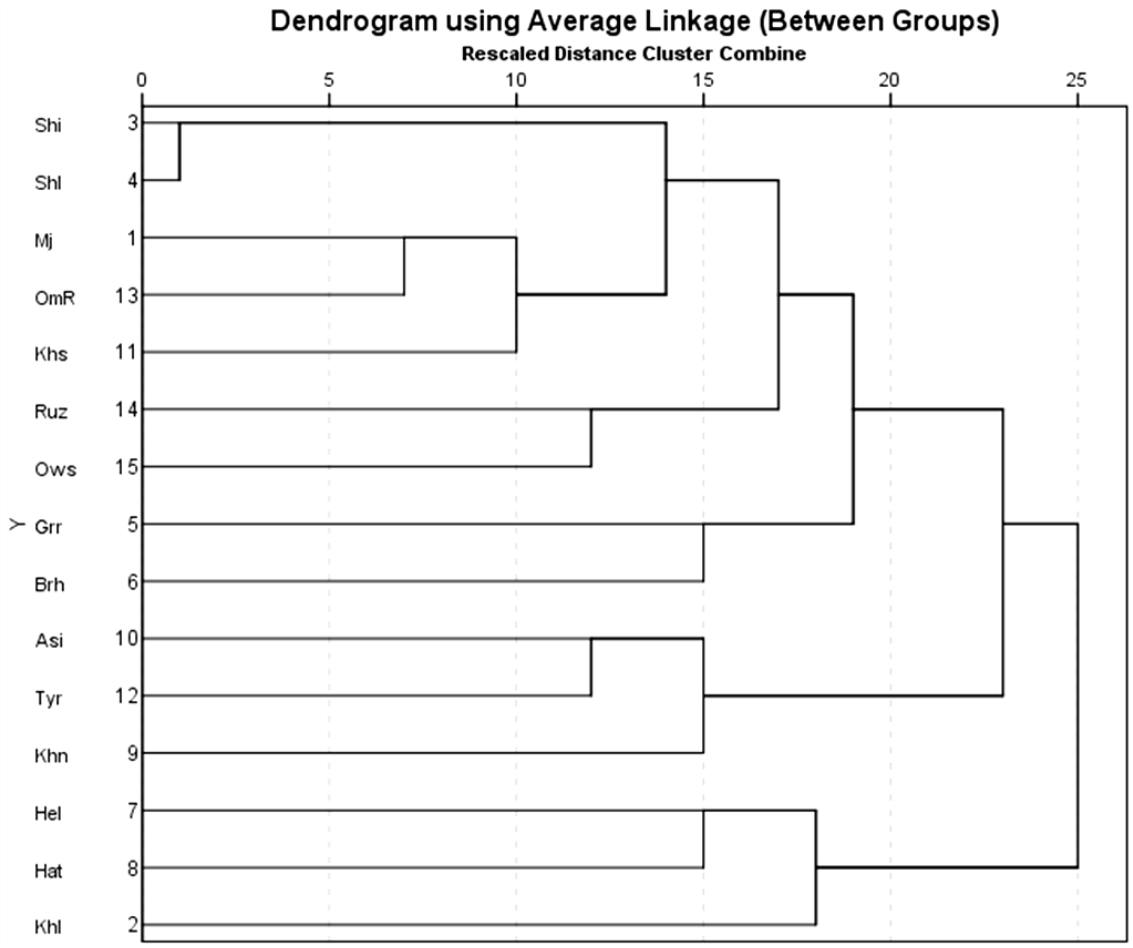

Figure. 3 Average linkage between groups hierarchical clustering dendrogram based on Jaccard`s Similarity showing genetic relationships among the different cultivars.

Table 4. The KMO and Bartlett`s tests for adequacy and sphericity.

Kaiser-Meyer-Olkin Measure of Sampling Adequacy.		0.722
Bartlett's Test of Sphericity	Approx. Chi-Square	46.550
	df	21
	Sig.	0.001

Table 5. Eigenvalues of 7 components, % of variance, % of cumulative variation for each component, first two components targeted.

Total variance explained									
Component	**Initial Eigenvalues**			**Extraction sums of squared loadings**			**Rotation sums of squared loadings**		
	Total	**% of Variance**	**Cumulative %**	**Total**	**% of Variance**	**Cumulative %**	**Total**	**% of Variance**	**Cumulative %**
1	3.661	52.296	52.296	3.661	52.296	52.296	3.162	45.167	45.167
2	1.100	15.712	68.008	1.100					
3	0.932	13.312	81.321						
4	0.738	10.542	91.863						
5	0.302	4.316	96.179						
6	0.174	2.485	98.664						
7	0.094	1.336	100.000						

Table 6. Displayed first two component represented by the variables and its loadings.

Rotated component matrix[a]		
	Component	
	1	2
B92	0.918	
B100	0.883	
B205	0.798	
B108	0.787	
B19		-0.773
B25		0.697
B37		0.597

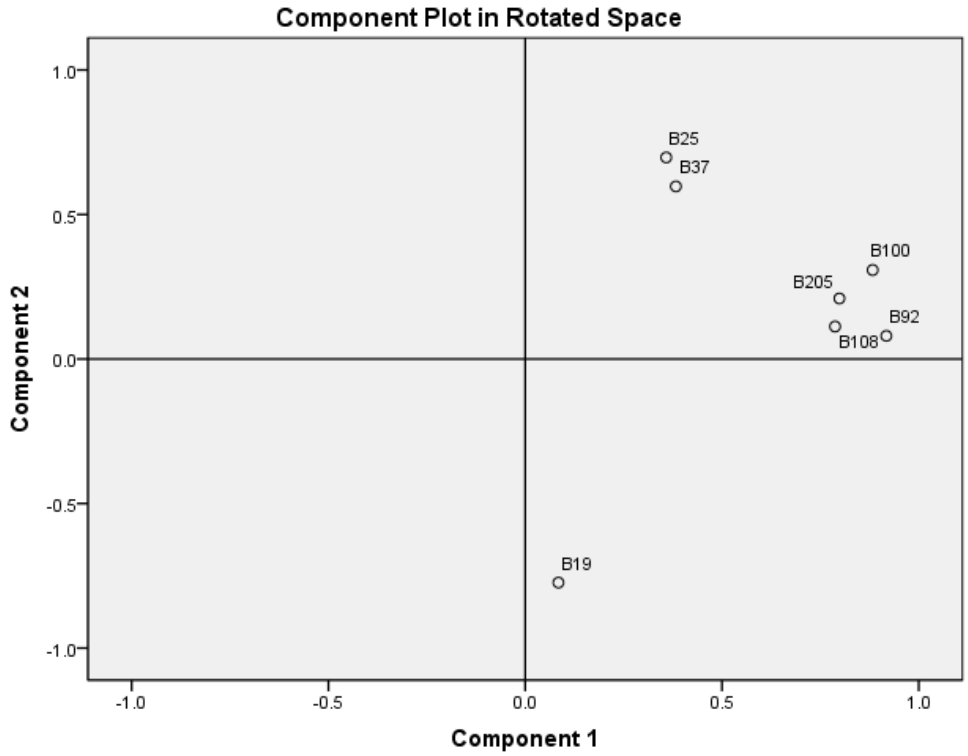

Figure. 4 Scatter diagram of electrophoretic bands according to the first two components.

be concluded that most of Al-Ahsa oasis cultivars, which were included in present study were refer to one genetic origin; such conclusions have been reported for other localities cultivars (Ahmed and Al Qaradhawi, 2009; Khierallah et al., 2014; Attaha et al., 2013; Khalifah et al., 2012), and agree with the conclusion, that the diversity of protein bands between varieties within species are generally low (Vaz et al., 2004; Hastuti and Prabang, 2009; Khalifah et al., 2012). The closely related cultivars "Hel" and "Hat" in addition to "Khl" cultivar which represent the sixth cluster, which separated out of other cultivars (Figure 3), with high degree of similarity which ranged between 0.636 to 0.714, (Table. 3), have been confirmed by the first principal component with high loading (52.3%) (Table 5), which was characterized by four bands 92, 100, 205 and 108 kda, (Table 6); these bands were mostly positioned close to each other in the scatter diagram (Figure 4). The second principal component, was characterized by low loading (15.7%) (Table 5) with three bands 25, 37 and 19 kda, (Table 6), where bands 25 and 37 were mostly positioned close to each other in the scatter diagram (Figure 4); it confirmed the first cluster of closely related cultivars "Shi" and "Shl" with high degree of similarity 0.917, as well as it confirmed

the closely related cultivars "Mj" and "OmR" among the second cluster, with high degree of similarity of 0.824.

The third cluster which is represented by closely related cultivars "Ruz" and "Ows" with high degree of similarity 0.750, (Table 3), have not been confirmed by either first or second components. The probable reason is that the cluster was not supported by enough distinguished bands being developed. Clustering of date palm cultivars by phylogenetic dendrograms and confirming this clustering by principal component analysis have been reported (Ahmed and Al Qaradhawi, 2009; Khoshroo et al., 2011; 2013; Attaha et al., 2013). The differences of protein bands intensity between cultivars protein patterns should be taken in the consideration too, since there are differences among studied cultivars in the intensity of protein bands, it appeared higher in "Hel" cultivar, lower in "Hat" cultivar, and also "Hel" and "Khn" cultivars seems higher in intensity of band 50 kda than other cultivars, as well as "Khl" and "Hel" cultivars seems higher in intensity of band 22 kda than other cultivars.

Protein banding pattern of "Brh" cultivar showed allelic variation at 239 kda, while "Grr" cultivar showed allelic variation at 46 kda; both were close related, sharing 12 common bands, with degree of similarity of 0.706, however, "Brh" cultivar was known as south Iraq originated cultivar (Al-Bakr, 1972), while "Grr" cultivar known as Al-Ahsa oasis originated (Asif, 1982); this close relation may be interpreted due to the gulf Arabic cultivars referred to one genetic origin.

Allelic variation have been shown in "Ows" cultivar at 165 kda, which was closely related to "Ruz" cultivar with 12 common bands, with degree of similarity 0.750, "Asi" cultivar showed allelic variation at 19 kda, which was closely related to "Tyr" cultivar with nine common bands, with degree of similarity 0.750, but although all allelic variations were resolved with low protein contents, they were regarded to the gene expression. However, the allelic variations could be used as an alternative, or complementary biochemical markers (Ould Mohammad et al., 2008; Abd El-Hady et al., 2010; Attaha et al., 2013). "Khs" and "Khn" cultivars which were known as Al-Qatif originated cultivars have been included in second and fifth clusters, respectively, however, this result in addition to "Brh" cultivar support previous conclusion that Arabic gulf cultivars were referred to one genetic origin, and they were distributed as a result of offshoots transportation within the Arabic gulf localities.

Conclusion

It could be concluded from the present study, even with the reality of the genetic diversity among date palm cultivars in Al Ahsa oasis that they belong to one genetic origin, since each cultivar was grown from a seed, however, the cultivar have been selected later due to preferred fruit characteristics. More and detail biochemical and molecular studies would be necessary to uncover the genetic relationships between date palm cultivars within Al-Ahsa oasis and other Arabic gulf localities.

Conflict of interests

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

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Influence of breeds genetic composition on the quality of milk from primiparous cows

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Forty-four crossbred primiparous cows participated in this study, namely: 15 1/2 animals corresponding to 1/2 Holstein (HO) x 1/2 Gyr; 15 3/4 animals corresponding to (3/4 HO x 1/4 Gyr) and 14 7/8 animals corresponding to (7/8 HO x 1/8 Gyr) raised under similar handling and feeding conditions. The sample collection was carried out between January and March 2013. To compare the quality of milk among breeds genetic compositions, the study was set up in a completely randomized design. Data of variables fat, protein, lactose contents, fat free dry matter (DDE), urea, somatic cell count, electrical conductivity, pH, titratable acidity and production were submitted to analysis of variance using the Tukey test at 5% probability for comparison of means between treatment 1 (1/2 HO x 1/2 Gyr), treatment 2 (3/4 HO x 1/4 Gyr) and treatment 3 (7/8 HO x 1/8 Gyr). Statistical analyses were performed using the SISVAR® - UFLA software. Correlation analysis between variables was performed using the ASSISTAT software. The results demonstrate that the genetic makeup of the different races holstein / Gyr primiparous cows did not affect the quality of the milk produced. The values of the chemical constituents of milk have been considered suitable for human consumption. Titratable acidity and milk production were higher for 3/4 Holstein/Gyr animals.

Key words: Protein, electrical conductivity, titratable acidity, Holstein, Gyr.

INTRODUCTION

In Brazil, the Holstein breed is the most exploited due to higher milk production compared with other breeds, especially in more intensive systems. According to Huang et al. (2009), the limiting factor for the use of Holstein cows is that the higher the milk production, the lower the reproductive potential. High reproductive efficiency is an important factor to ensure profitable milk production.

Another aspect that has generated concern, particularly

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in Holstein herds of high productivity is the reduced productive efficiency in dairy herds caused by climatic factors (Almeida, 2007).

Gyr animals are adapted to tropical conditions and show lower incidence of diseases than breeds in temperate climates (Van Melis et al., 2007). The Gyr is one of the principal Zebu breeds, these breeds play an important role in the Brazilian dairy farming (Silva, 2012).

The Gyr breed stands out in relation to other breeds of Indian origin for the excellent productive and reproductive performances, which are associated with rusticity, good milk production performance, for manual as well as mechanical milking. A viable alternative is the crossing between breeds of Indian (Zebu) and breeds of European origin (Taureans), which combines the tropical climate, Indian breeds with the productive potential of taurine breeds (Vasconcellos et al., 2003). The crossing between Holstein and Gyr animals is the origin of the Girolando breed, a rustic animal adapted to the tropical climate and adequately efficient in milk production.

One of the frequent concerns in dairy farming is the order of deliveries of dairy cows. Soares et al. (2009) reported that the average milk production of adult cows (above 4 births) is higher than first- and second-order cows, which improves the economic value of the herd.

The high production of Holstein milk and the highest concentration of solids in the milk of animals of Zebu, is a factor to be considered for choosing the cross, that is, the Dutch Animal produce more milk with lower solids content, as the zebu cows produce less milk with higher solids content. Thus, the cross becomes indispensable, because we can gather the two characteristics in the same animal.

The production efficiency of cows with different breeds genetic compositions (Holstein/Gyr) is still unknown. There is a need to seek optimization of the productivity and quality of milk from cows that originated from the crossing of cows of European and Indian origins, justifying the conduction of studies in this area. Therefore, the aim of this study was to evaluate the physicochemical composition, production and somatic cell count (SCC) of milk from primiparous cows with different breeds genetic compositions (Holstein/Gyr).

MATERIALS AND METHODS

The study was conducted during the rainy season, between January and March 2013, on a cattle farm located in Rio Verde - Goiás, Brazil (17 56 '3:38 "S and 5 ° 2' 3.85" W) . The average temperature during the experimental period was 32°C and relative humidity of 37%. The region has climate with two distinct seasons: the dry season (May to October) and rainy (November to April).

The herd consisted of 140 crossbred lactating cows, producing approximately 19.22 liters/cow/day. The animals used in the experiment had average age of 36 months, weighing between 350 kg and 490 kg in stage of lactation between 90 and 110 days.

The study included 15 animals mestizo 1/2 blood, that is F1 (50% of Holstein x Gyr 50%), 15 animal crossbred 3/4, that is, F2 (75% of Holstein x Gyr 25%) and 14 crossbred animals 7 / 8, or F3 (87.5% of Holstein x Gyr 12.5%), raised in similar handling and feeding conditions. The cows received all mandatory vaccinations (foot-and-mouth disease, brucellosis and anthrax) in accordance with recommendations of the veterinary and according to requirements of the vaccine schedule set by the Board of Agriculture and Livestock Defense of the State of Goiás.

Table 1. Chemical composition of *Brachiaria brizantha* cv Marandu pasture offered to lactating cows.

Composition	(%)	SD
Dry matter	28.33	1.72
Mineral matter	7.99	0.14
Crude protein	16.01	0.36
Neutral detergent fiber	55.75	2.68
Acidd etergent fiber	33.68	1.94
Ether extract	2.80	0.36
Total digestible nutrients	67.39	1.82

Table 2. Chemical composition of the concentrate offered to primiparous lactating Holstein/Gyr cows.

Composition	(%)	SD
Dry matter	89.08	1.78
Mineral matter	6.39	0.45
Crude protein	22.12	0.22
Neutral detergente fiber	26.32	0.60
Acid detergent fiber	18.26	0.83
Ether extract	4.51	0.25
Total digestible nutrients	87.40	1.04

For the development of this study, the dairy cows grazed on intensively managed *Brachiaria brizantha* cv Marandu pasture, whose chemical composition is shown in Table 1.

Cows had free access to the resting area with natural and artificial shade, water and mineral salt *ad libitum*. The assessment of the nutritional value of forage was performed after collection of samples, using a metal square with an area of 1 m^2. The square area was released at random in the area for measurement of pasture height with the aid of a ruler. The forage samples were collected once a week throughout the experimental period (January-March 2013). Shortly after, the material was collected with the aid of pruning shears, and then the samples were placed in properly labeled plastic bags for later analysis. Commercial protein concentrate was daily provided to cows in lactation at a ratio of 1 kg of concentrate per 4 L of milk produced (Table 2).

The assessment of the nutritional components of commercial protein concentrate was performed after collection of samples directly obtained from the storage bags. The concentrated samples were collected once a week during the entire experimental period (January-March 2013). The collected material was placed on properly labeled plastic bags. Then, a representative sample was collected for chemical composition assessment.

Dry matter (DM) of fodder and concentrate were determined in an oven with forced air circulation at 55°C. Subsequently, the samples were ground in a 1 mm sieve for analysis.

DM, crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), ether extract (EE) and mineral matter (MM)

Table 3. Mean values and standard deviation of fat (%), protein (%), lactose (%), fat free dry matter defatted dry extract (%), urea (mg/dL), somatic cells count (x1000 SCC/mL), electrical conductivity (mS/cm^2), pH and titratable acidity (g of lactic acid/100 mL) of milk from Holstein/Gyr cows.

Parameter	Breeds genetic composition Holstein/Gyr			VC CV (%)	p-value
	½ (n=75)	¾ (n=75)	7/8 (n=70)		
Fat	3.33 ±0.59	3.27 ±0.60	3.39 ±0.68	18.73	0.4895
Protein	3.16 ±0.35	3.07 ±0.27	3.12 ±0.35	10.51	0.2186
Lactose	4.57 ±0.31	4.59 ±0.22	4.58 ±0.20	5.44	0.8704
DDE FFDM	8.76 ±0.42	8.66 ±0.40	8.70 ±0.34	4.44	0.3140
Urea	17.77 ±3.16	18.13 ±5.19	18.73 ±4.93	24.77	0.4333
SCC	382 ±538	293 ±455	460 ±655	147.02	0.1959
EC	4.89 ±0.49	4.81 ±0.35	4.80 ±0.31	8.09	0.3274
pH	6.35 ±0.45	6.30 ±0.53	6.30 ±0.50	7.82	0.7804
TA	0.163 ±0.018ab	0.167 ±0.020a	0.159 ±0.019b	11.20	0.0290

Means followed by different letters in the row differ significantly ($p< 0.05$) in accordance with 5% Tukey test. n = number of samples collected. VC CV = variation coefficient. DDE = defatted dry extract; FFDM = fat free dry matter; SCC = somatic cells count; EC = electrical conductivity;TA = titratable acidity.

of fodder and concentrate were determined as described by Silva and Queiroz (2002).

Total digestible nutrients (TDN) were determined using the following mathematical equation: TDN (%) = 105.3 - (0.68 x % NDF), according to NRC (1996).

Cows were milked twice a day; the first milking was performed at 6:00 am and the second at 4:00 pm. The production of animal milk used in the study were measured only at baseline.

Milk samples of lactating cows were collected once a week for a period of three months (January-March 2013), at the first milking of the day. Milk samples were always collected from the same animals throughout the experimental period.

At the time of milking, the first three jets of milk were collected in a mug of black bottom for detection of clinical mastitis, positive animals were not milked.

Milk of each cow was collected in individual milk meters provided with a valve. Before collection, the milk was stirred for 5 s. Then, the milk sample was taken by positioning the valve in the emptying option, transferring the content to the milk collector.

Sterilized bottles with capacity of 40 ml containing Bronopol® were used for analysis of chemical composition and somatic cell count (SCC). All bottles were previously identified with labels containing barcodes corresponding to the code of each animal. Milk volume (L), of each animal was measured at the beginning of the experimental period.

After collection, milk samples were stored in isothermal boxes containing ice and sent for analyses to the Laboratory of Milk Quality - Food Research Centre, School of Veterinary and Animal Science, Federal University of Goiás.

The fat, protein, lactose and defatted dry extract (DDE) fat free dry matter content were determined using the MilkoScan 4000 equipment (Foss Electric A/S. Hillerod Denmark). According to IDF (2000); the results were expressed as mass percentage (%); urea contents were determined by differential Fourier transform infrared spectroscopy (FTIR) using Lactoscope equipment (Delta Instruments). The results were expressed as mg/dL. Somatic cell counts (SCC) were determined by flow cytometry according to IDF (2006); results were expressed as SC/mL. The electrical conductivity of milk was determined using a conductivity meter TECNOPON® model mCA - 150, with results expressed as mS/cm^2. pH was measured using bench microprocessor W3B pH meter from Bel Engineering®. Acidity was determined by titration according to the method of AOAC (1995) and the results were expressed as grams of lactic acid per 100 mL of milk.

The study was set up in a completely randomized design (CRD). For comparison of the milk quality between breeds genetic compositions of cows, data of variables fat, protein, lactose contents, fat free dry matter DDE, urea, SCC, electrical conductivity, pH, titratable acidity and production were analyzed by ANOVA using the Tukey test at 5% probability. For comparison of means between treatments, statistical analyses were performed using the SISVAR® - UFLA software. Correlation pearson analysis between variables (breeds, production, fat, protein, lactose contents, fat free dry matter, urea, SCC, electrical conductivity, pH, titratable acidity) was performed using the ASSISTAT software (Silva and Azevedo, 2009).

RESULTS AND DISCUSSION

The average results and standard deviations of the physicochemical composition and SCC of milk from primiparous Holstein /Gyr crossbred cows are shown in Table 3.

According to the values in Table 3, no significant differences ($p>0.05$) in milk fat content between breeds genetic groups were observed. The variation coefficient (VCCV) was 18.73%. The fat content of samples ranged from 3.27 to 3.39% (Table 3). The results were above the limit established by Brazilian legislation, which recommends a minimum fat value of 3.0% (Brasil, 2011). These results were lower than those observed by Lima et al. (2006), who found fat contents from 3.34 to 3.56% for C-type pasteurized milk produced in the semiarid region of the state of Pernambuco.

Better results than those obtained in this study were reported. Higher results than those obtained in this study have been reported by Mendes et al. (2010), who determined fat contents up to 3.8% when evaluating the quality of cow's milk sold in the city of Mossoró, RN, Brazil.

Varying amounts of nutrients daily ingested by lactating

cows can cause oscillations in the major milk components such as fat, protein and lactose. We can observe that milk fat values were close to the protein, explaining that animals consume a larger amount of concentrate, or roughage to concentrate in the diet is low, increasing milk protein content and decreasing the fat content.

There was no significant difference ($p>0.05$) in the protein content of milk between breeds genetic groups, and the variation coefficient (VC)CV was 10.51%. The average protein values of milk from primiparous Holstein x Gyr cows ranged from 3.07 to 3.16%. These results were higher than those proposed by Normative Instruction 62/2011, which calls for minimum levels of crude protein 2.9%. Values similar to those obtained in this study were observed by Mota et al. (2008), who assessed production performance and milk composition of Holstein cows in late phase of lactation and found protein contents ranging from 2.97 to 3.16%.

An important factor that influences the protein content in milk is the phase of lactation of cows. Lactating cows aged over seven years tend to produce milk with higher protein content and compared to first-parity cows (Noro et al., 2006).

To investigate production and quality of milk from Holstein cows according to the parity order, Souza et al. (2010) found no significant effect of the number of lactation on protein content, with mean value of 3.23%.

The lactose results did not differ significantly, with average values of 4.57, 4.59 and 4.58%, for primiparous Holstein/Gyr, 1/2, 3/4 and 7/8 cows respectively. Regardless of breed genetic composition, lactose was synthesized by the mammary gland of cows in similar amounts.

Lactose results similar to those obtained in the present study (4.42%), were found by Botaro et al. (2011) in a study aimed at evaluating the composition and protein fraction of milk from commercial herds in the state of São Paulo, while Fukumoto et al. (2010) reported an average of 4.2% lactose when assessing milk production and composition, dry matter intake and stocking rate in tropical grass pasture managed under rotational grazing system.

The fat free dry matter content defatted dry extract (DDE) did not differ significantly ($p>0.05$) between breeds genetic groups, and the VC CV was 4.44%. The values were 8.76% for milk from1/2 primiparous cows; 8.66 % for milk from 3/4 cows and 8.70% for milk from 7/8 cows. These results were consistent with the IN 62/2011 values, which establishes minimum fat free dry matter DDE value of 8.4% in milk.

DDE fat free dry matter contents values similar to those obtained in this study have been reported by Cerdótes et al. (2004), which ranged between 8.55 and 8.75% when investigating the production and composition of milk from cows of four breeds genetic groups submitted to two feeding managements.

The urea concentrations in milk produced by breeds genetic groups 1/2, 3/4 and 7/8 were 17.77, 18.13 and 18.73 mg/dL, respectively, and no significant difference ($p>0.05$) in the urea levels among these groups was observed. The CV variation coefficient (VC) was 24.77%.

According to Wang et al. (2007), increased urea levels in milk are influenced by metabolizable protein present the diet offered to lactating cows. Fluctuations in the crude protein levels in the diet and the way it is provided to lactating cows considerably alter the concentrations of ruminal ammonia and consequently the level of nitrogen in the form of urea in blood and also in milk (Hojman et al., 2005).

Urea levels below 12 mg/dL and above 18 mg/dL may result from improper nutrition management. Based on the chemical analyses of concentrate and fodder, (Tables 1 and 2), concentrate and fodder were excellent protein sources for lactating cows.

Aquino et al. (2007) observed urea concentrations in milk ranging from 16.59 to 17.97 mg/dL when studying the effect of increasing urea levels in the diet of dairy cows on milk production and physicochemical composition.

The average SCC did not differ significantly ($p>0.05$). The variation coefficient CV was 147.02%, with values of 382,000, 293.000 and 460.000 SC/mL, obtained from primiparous Holstein/Gyr, 1/2, 3/4 and 7/8 cows, respectively. These values are below limits established by Brazilian legislation. The high variation coefficient of the SCC was due to the large amplitude of counts, whose standard deviations were 538.000, 455,000 and 655,000 SC/mL for the three breeds genetic compositions studied.

The SCC of milk should not exceed the limit of 600,000 SC/mL of milk, as described in the Normative Instruction IN 62 (Brasil, 2011) for the period from January 1, 2012 to June 30, 2014, in the region under study, which is the southwestern state of Goiás, located in the Mid-western region of Brazil.

The gradual increase in somatic cells count causes a decline in production and influences the physicochemical characteristics of milk, in enzymatic activity, coagulation time, yield and quality of dairy products (Arashiro et al., 2006).

Cunha et al. (2008) evaluated the relationship between subclinical mastitis and SCC with number of lactations, production and chemical composition of milk from Holstein cows and observed that animals with larger number of lactations had higher SCC values and cows that had SCC above 100,000 SC/mL had lower milk production.

Besides SCC, there are other features also related to the occurrence of mastitis, among those changes in the concentration of anions and cations in milk, which is determined by the electrical conductivity and deserves attention for being a relatively easy and inexpensive method for the diagnosis of subclinical mastitis (Zafalon et al., 2005).

Table 4. Milk production variation of primiparous Holstein/Gyr cows.

Parameter	Breeds genetic composition Holstein/Gyr			VC CV (%)	P-value
	½ (n=75)	¾ (n=75)	7/8 (n=70)		
Milk production (L)	18.41 ±3.24b	20.25 ±2.92a	19.01 ±3.32b	16.44	0.0016
Mean production (L)	19.22				

Means followed by different letters in row are statistically different ($p < 0.05$) by the Tukey test. n = number of samples collected. VC CV = variation coefficient.

Electrical conductivity measures the ability of a solution to conduct electrical current between two electrodes and is given in milliSiemens per centimeter (mS/cm).

As described in Table 3, no significant difference ($p>0.05$) for the average electrical conductivity values between the breeds genetic groups was observed, with values of 4.89, 4.81 and 4.80 mS/cm, obtained from primiparous Holstein/Gyr, 1/2, 3/4 and 7/8 cows, respectively. The CV was 8.09%.

The electrical conductivity of milk ranges from 4.61 mS/cm to 4.92 mS/cm (Ferreira, 2007), therefore, the means of this study are within the allowable limit for EC. According to Santos (2005), the EC values of milk increase to 5.37 mS/cm in subclinical cases and to 6.73 mS/cm for clinical cases of mastitis.

In a study by Della Libera et al. (2011) evaluating methods for detection of mastitis, the results indicated that when used for this purpose, the EC must be used with caution, because this parameter may be influenced by age, stage of lactation, production, season of year, milk fraction collected and pathogenic agents.

According to Zafalon et al. (2005), major changes in the physicochemical characteristics of milk occur in cows with mastitis, especially regarding electrical conductivity and pH.

pH has great importance in milk technology because all fermentative phenomena, processes of butter formation, protein precipitation and pasteurization results depend on milk pH (Ferreira, 2007).

According to the results in Table 3, average pH values of 6.35, 6.30 and 6.30, corresponding to primiparous Holstein/Gyr, 1/2, 3/4 and 7/8 cows, respectively, did not differ ($p> 0.05$). The high SCC milk, demonstrating that animals showed subclinical mastitis regardless of the race, that is, these microorganisms present in the milk directly influenced pH drop.

Average values for itratable acidity were significantly different ($p<0.05$) between breeds genetic groups, 3/4 Holstein/Gyr cows produced milk with higher acidity (0.167 g of lactic acid/100 mL) compared to 1/2 and 7/8 cows (0.163 and 0.159 g of lactic acid/100 mL, respectively).

According to Normative Instruction N^{o} 62 (Brasil, 2011), bovine milk is considered of good quality when showing acidity values between 0.14 and 0.18 g of lactic acid/100 mL of milk, which can be evidenced in this study because all breeds genetic groups evaluated showed normal values.

According to Santos and Fonseca (2006), soon after milking, milk has a slightly acid reaction due to some of the components. This acidity, called natural or apparent, is caused by albumin (1st D), citrate (1st D), carbon dioxide (1st D), caseins (5th D and 6th D) and phosphate (5th D).

The results of physicochemical parameters of milk from primiparous Holstein/Gyr, 1/2, 3/4 and 7/8 cows, indicated good quality and compliance with standards established by law.

The mean estimates of daily milk production of different breeds genetic groups areshown in Table 4, expressed as liters of milk/day.

Among the genotypes evaluated, 3/4 Holstein/Gyr animals were more productive than in 7/8 and 1/2 animals, and this result can be partly explained due to the condition of primiparous cows, which are still under development, being one of the variables that affect production (Coffey et al., 2006).

Lower results were described by Vilela et al. (2007) evaluating Holstein cows in lactation up to 200 days grazing on coast-cross grass and supplemented with 3 kg or 6 kg concentrate/cow/day, with production of 15.5 kg and 19.1 kg of milk/cow/day. Milk production values higher than those obtained in this study were reported by Silva et al. (2011) evaluating the production of milk from multiparous Holstein cows of small, medium and large size, with values from 30.56 to 31.07 kg/milk/day and average of 23.27 kg.

Glória et al. (2006) evaluated the effects of breeds genetic composition and environmental factors on milk production of Holstein-Gyr crossbred cows and observed that the breeds genetic composition directly reflects the increase in total milk production with increased contribution from Holstein breed.

The linear correlation results between breeds, milk volume produced, SCC, electrical conductivity, pH, titratable acidity and chemical components of milk are presented in Table 5.

There was no significant linear correlation ($p>0.05$) between breeds genetic composition and milk production, content, protein, lactose, fat free dry matter DDE, SCC, urea, EC, pH and acidity, indicating that blood degree did not influence the physicochemical characteristics of milk

Table 5. Linear correlation between quality variables and milk production of crossbred Holstein/Gyr cows.

Parameter	Production	Fat	Protein	Lactose	DDEFFDM	SCC	Urea	EC	pH	TA
GC Breeds	0.12^{ns}	0.02^{ns}	-0.07^{ns}	0.01^{ns}	-0.08^{ns}	0.03^{ns}	0.08^{ns}	-0.10^{ns}	-0.04^{ns}	-0.05^{ns}
Production	-	0.08^{ns}	-0.02^{ns}	-0.22**	-0.17*	-0.09^{ns}	0.09^{ns}	0.03^{ns}	0.09^{ns}	-0.07^{ns}
Fat	-	-	0.28 **	-0.14*	0.19**	0.08^{ns}	-0.05^{ns}	-0.11^{ns}	-0.01^{ns}	-0.07^{ns}
Protein	-	-	-	-0.17*	0.75**	0.35**	0.29**	0.04^{ns}	0.17*	-0.07^{ns}
Lactose	-	-	-	-	0.48**	-0.47**	0.21**	-0.54**	-0.11^{ns}	0.36**
DDE FFDM	-	-	-	-	-	0.03^{ns}	0.39**	-0.30**	0.06^{ns}	0.15*
SCC	-	-	-	-	-	-	-0.02^{ns}	0.32**	0.13^{ns}	-0.26**
Urea	-	-	-	-	-	-	-	-0.14*	-0.03^{ns}	0.03^{ns}
EC	-	-	-	-	-	-	-	-	0.09^{ns}	-0.16*
pH	-	-	-	-	-	-	-	-	-	-0.12^{ns}

**Significant at 1% probability level (p<0.01). * Significant at 5% probability (p<0.05). ns = not significant (p≥0.05). T-test was applied levels of 5% and 1%. GC = genetic composition; DDE = defatted dry extract; FFDM = fat free dry matter contents; SCC = somatic cells count (x1000 SC/ml); EC = electrical conductivity; TA = titratable acidity.

from primiparous Holstein/Gyr cows. In addition, there was no linear correlation between milk production and fat, protein, SCC, urea, EC, pH and acidity of milk from primiparous cows.

In Brazil, milk production and fat content are the most productive features for dairy industries in relation to milk payment. The protein content is extremely important, especially for the manufacture of cheese and other dairy products, since protein is determinant of the yield of the final product.

The linear correlation between fat and protein content (r = 0.28) of milk produced by primiparous Holstein/Gyr cows was significant and positive. The higher the fat content of milk produced by crossbred cows, the higher the protein content.

There was a negative correlation at 5% probability (p<0.05) between fat and lactose (r = -0.14), indicating that the higher the fat content of milk produced by primiparous cows, the lower the lactose content.

The linear correlation between fat content and fat free dry matter DDE (r = 0.19) was positive at 1% probability (p<0.01), showing that the higher the fat content of milk, the higher the fat free dry matter DDE levels, corroborating the results of Oliveira et al. (2010), who evaluated the physicochemical composition of milk at different stages of lactation and found that the fat content, fat free dry matter DDE and lactose were the variables that most varied during the lactation period.

The correlation between fat content and SCC (r = 0.08), urea (r = -0.05), electrical conductivity (r = -0.11), pH (r = -0.01), and titratable acidity (r = -0.07) was not significant (p>0.05).

Different results were reported by Santos, (2005), who observed a negative correlation between EC and fat content, because fat has low capacity to conduct current. According to Rodrigues, (1998), the increased fat content inhibits the EC not only due to the reduction of ions, but also to the physical barrier that fat globules represent for them.

The linear correlation between protein and lactose was negative at 5% probability (p<0.05) (r = -0.17); these results suggest that the higher the protein content of milk produced by primiparous cows, the lower the lactose content.

The linear correlation between protein content and fat free dry matter DDE (r = 0.75), SCC (r = 0.35), urea (r = 0.29) and pH (r = 0.17) was positive. Under the conditions of this study, increased protein contents has led to a progressive increase in defatted dry extract fat free dry matter, urea, SCC and pH and milk produced by primiparous cows, corroborating the results obtained in this study. Cunha et al. (2008) observed positive correlation between SCC and protein content of milk from Holstein cows.

Ventura et al. (2006) evaluated the SCC and the effects on milk constituents and found that a minimal increase in the protein content led increase in the SCC values with correlation of 0.2563.

There was no significant correlation (p≥0.05) between protein content, EC and titratable acidity of milk.

There was a positive correlation (p<0.01) between lactose, fat free dry matter DDE (r = 0.48), urea (r = 0.21) and titratable acidity (r = 0.36) and negative for SCC (r = -0.47), electrical conductivity (r = -0.54) and pH (r = -0.11), the latter being not significant (p≥0.05). This shows that the greater the synthesis of lactose, the greater fat free dry matter content DDE, urea, and TA and the lower the SCC and EC in milk, thus indicating the large number of cows affected with mastitis.

Rajcevic et al. (2003) observed a significant negative correlation between SCC and lactose content (r = -0.42). On average SCC is high, thereby affecting on the correlations, making them significant.

The linear correlation between SCC, fat free dry matter

DDE and pH was not significant (r = 0.03) and (r = 0.06), respectively. The linear correlation of fat free dry matter DDE with urea (r = 0.39) was positive and significant at the 1% level of probability (p<0.01), negative and significant at 1% with electrical conductivity (r = 0.30), positive and significant (p<0.05) with titratable acidity (r = 15).

The correlation between SCC and electrical conductivity (r = 0.32) of milk from primiparous Holstein/Gyr cows was positive and significant at 1% probability (p<0.01). The increase in electrical conductivity of milk is directly proportional to increased inflammation of the udder and SCC. Tavares and Rodrigues, (2010) used the electrical conductivity of milk to assess the health of the udder of dairy cows and found a correlation between EC and SCC of 0.257; and according to the author, the correlation between EC and SCC is in general positive.c

The correlation between SCC, urea and pH was not significant (p≥0.05), but the linear correlation between SCC and titratable acidity was negative but significant at 1% probability (p<0.01).

There was no correlation (p≥0.05) between urea, pH (r = -0.03) and titratable acidity (r = 0.03). The correlation between urea and EC (r = -0.14) was negative and significant at 5% probability (p<0.05), but significantly decreased the electrical conductivity of milk.

There was no significant linear correlation (p≥0.05) between EC and pH (r = 0.09). The correlation between EC and titratable acidity of milk from primiparous cows was negative and significant (p<0.05). This demonstrates that the electrical conductivity does not change the pH but negatively influence the titratable acidity of milk from Holstein/Gyr cows.

Due to the fact that milk has electrolytes that favor the passage of electric current, the electrical conductivity values of milk can be used to detect abnormal milk, that is, with subclinical mastitis (Zafalon et al., 2005).

Santos (2005) reported that this type of mastitis diagnosis can reach around 80% sensitivity (correct identification of infected cows) and 75% specificity (correct identification of healthy cows), that is the use of EC to determine SCC is reliable. However, for greater accuracy, association with SCC results is required.

There was no significant linear correlation between pH and titratable acidity (r = -0.12). The difficulty of obtaining a good correlation is related to the fact that in determining the acidity, free (ions) and accessible (ionizable/ dissociable) hydrogen protons are measured, on the other hand, only free hydrogen protons (ions) are quantified in pH determination (Silva, 2004).

Primiparous cows originated from the crossing between Holstein and Gyr stood out for efficient production. Accordingly, it was observed that the milk produced had met the quality requirements set by Brazilian legislation.

Although the values of the chemical components of milk are in accordance with the values required by Brazilian law, it should be noted that SCC possesses high positive correlation with EC, showing that some animals have subclinical mastitis, with that, the dairy derivatives from milk with high SCC can have their shelf life and their low income, directly influencing the quality of the final product.

Conclusion

The blood degrees of primiparous Holstein/Gyr cows did not influence the chemical quality of milk. The values of the chemical constituents of milk were considered suitable for human consumption. Titratable acidity and milk production were higher for 3/4 Holstein/Gyr animals.

Conflict of interests

The authors did not declare any conflict of interest.

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