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

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Table of Contents: Volume 13 Number 11, 12 March, 2014

ARTICLES

Table of Contents: Volume 13 Number 11, 12 March, 2014

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Review

Recommended temperature and relative humidity for storage of Brazilian tropical flowers

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Post-harvest treatment and proper handling is essential so that the flowers can maintain their quality during marketing and exporting. This review aims to gives information on the storage temperature used in postharvest handling of tropical flowers of Brazil. This review provides detailed specifications and recommendations for storing the six main Brazilian tropical flowers.

Key words: Floriculture, refrigeration, quality assurance, conservation.

INTRODUCTION

Cut flowers slowly deteriorate and lose quality after harvest. Good postharvest treatment slows the loss of quality. Quick handling makes the flowers get to customers while they still look fresh. The stem of flowers need clean water and the leaves need humid and cool air in order to prevent wilting. Re-cutting of the base of the stem or addition of hydrating treatments to the water, or both often improve water uptake. Cold handling dramatically delays quality loss, water loss and death; postharvest solutions can improve water uptake, delay ageing and deterioration and improve flower opening; ethylene protection for sensitive flowers will reduce flower drop and ageing. Exposure to ethylene should sometimes be avoided, and the action of ethylene can be slowed or stopped by anti-ethylene treatments. Pest control kills insects that would otherwise lead to rejection of flowers in the market place or fumigation at the growers' or exporters' expense. Fungicide treatment may be needed to control fungal growth on some flowers; packaging enables efficient transport, protects flowers physically, and keeps flowers cool and humid (Faragher et al., 2010). In summary, we will only cover recommended temperatures for some species of tropical flowers in Brazil.

ANTHURIUM (*Anthurium andraeanum* Lindl)

The ideal storage temperature for anthurium is 15-18°C and RH is 90 to 95%; below 10 to 12°C, damage occurs, and the sheaths are bluish or wilted. High humidity favors the extension of vase life, and conserves water loss through transpiration (Figure 1). The flowers must be transported, for ease of packing in refrigerated trucks (isothermal) to the airport. In all cases, care should be taken regarding contact with wind, sun and rain (Paull, 1987; Lamas, 2004).

HELICONIA (*Heliconia* spp)

Arriving at the packing house, the stems should be placed in clean water. This practice increases durability by helping to reduce the temperature of the same, and

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Figure 1. Anthurium yellow.

Figure 2. *Heliconia psittacorum.*

wipe away dirt and remove the bad smell of some species. Heliconia flowers may be intended for different uses (Figure 2). Because this classification is important, care should be taken to select them by the number of bracts opened; usually a pointer and a five bracts open. It is recommended that before packaging, the stem should be cut and submerged in water containing chlorine solution of 0.02%; this acts as bactericidal. In the handling and packaging of inflorescences of heliconia, just as other flowers, special care is required to avoid injuries and damage to the same. The ideal temperature for handling heliconia is between 17 and 19°C and

Figure 3. *Strelitzia reginae*.

storage is above 14°C. In practice, given the susceptibility to low temperature is to write in the boxes, "not refrigerated" (Castro, 1998; Lamas, 2000; 2004).

STRELITZIA (*Strelitzia reginae* Banks ex Aiton)

In *Strelitzia reginae* (Figure 3), the biggest problem in the post-harvest is incomplete opening of the flowers and an apparent susceptibility to chilling injury (Macnish et al., 2009) besides the development of spots on the inflorescences few days before harvest (Pizano, 2005; Hassan, 2009). The ideal temperature for handling the inflorescences of Strelitzia is above 7°C with closed button: 1 month to 7°C; 85-90% RH. Vieira (2011) studying two temperatures of cold storage to Strelitzia noted the temperature of 7.5 showed symptoms of necrosis in the inflorescences being recommended temperature of 10 º C.This author also noted that 10 º C is not recommended for cut chrysanthemum FAROE (Vieira and Lima, 2009).

RED GINGER (*Alpinia purpurata* (Vieill.) Schum)

Upon arrival at the treatment shed (packing house), the stems should be placed in clean water; this practice increases durability by helping to reduce the temperature of the same and facilitate cleaning. The remaining leaves should be removed. The leaves of the stems should be removed and flushed to the pseudostem; the two sheets terminals can be left. This facilitates packaging, as it protects the leaves from injury. As for the procedures, after cutting in the field, the stems are always immersed in water; these inflorescences present a durability of at least 15 days or so. The pH of the water during post-harvest handling should be in the range of 4.5. The sizes

Figure 4. *Alpinia purpurata.*

Figure 5. *Etlingera elatior*.

of rods for sale range from 0.60 to 1.10 m, including the inflorescence. The classification of alpinias takes the size of inflorescences in the following classes: the transport and storage of Alpinia need special care, depending on their sensitivity to cold and dehydration. The ideal storage temperature for red ginger and transport in refrigerated environment is 15-18°C and high relative humidity (Figure 4) (Atehortua, 1998; Castro, 1998; Lamas, 2000; 2004).

Figure 6. *Zingiber spectabile.*

TORCH GINGER (*Etlingera elatior* R.M. Smith)

Since harvest is in the field, stems should be immersed in water. At the packing house, the rods must be immersed in pure water, preferably lying without the need for immersing the inflorescence. The inflorescences have a durability of approximately 15 days. The transport and storage of torch ginger need special care due to their sensitivity to cold and dehydration (Figure 5). The ideal temperature for handling the inflorescence is between 17 and 19°C and storage is above 14°C (15 - 18º C). High relative humidity needs to be maintained (90-95%). In practice, given the susceptibility at low temperature is to write in the boxes, "not refrigerated" (Castro, 1998; Lamas, 2004).

BEEHIVE GINGER (*Zingiber spectabile*, Griff)

At the packing house, the stems should be placed in clean water. This practice increases durability by helping to reduce the temperature of the same, and clean the soil. After cleaning and drying, they must be individually wrapped, protected by mesh or plastic bags. The boxes, with unit packets, must be lined with polyethylene film. When properly handled and prepared, the inflorescences of beehive ginger have a shelf life of up to three weeks (Figure 6). It is recommended that before packaging, the stem should be cut and submerged in water containing

bactericidal solution. The ideal storage temperature for beehive ginger and transport in refrigerated environment is 15-18°C and high relative humidity. When exposure occurs at low temperatures, the inflorescences present early wilting and browning of the bracts, besides favoring dehydration (Leitão, 2000; Lamas, 2004).

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Full Length Research Paper

Molecular discrimination and genetic relationships between some cultivars of *Cucurbita pepo* ssp. *pepo* using random amplification of polymorphic DNA (RAPD) analysis

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***Cucurbita pepo* ssp. *pepo*; zucchini group is a widely grown and economically important group belonging to genus *Cucurbita*, and being one of the easiest groups to cultivate in temperate climate with overwhelming production. Since, RAPD analysis provides a fast and reliable method for molecular characterization and investigation of the intraspecific genetic relationships; it had been used in this study to discriminate and clarify the genetic diversity between seven cultivars of *C. pepo* ssp. *pepo* originated worldwide. Seven out of 20 decamer arbitrary primers showed polymorphism in the RAPD profile. The polymorphism was investigated by 87 consistent amplification products. Some of those fragments were uniquely amplified in single cultivar. Thus, they can be used as molecular markers for cultivar identification. The results of Jaccard similarity and the phenogram ascertained the wide genetic base of the Egyptian landrace El-Escandrani with the lowest loading component of 0.400. Therefore, it could be recommended as a reservoir of alleles useful for breeding programs in parental crosses. The multivariate analysis using the principle component analysis separated all the cultivars on the first component indicating the high correlation between them. The strongest correlation was confirmed between the two MHTSQ hybrids with Mansoura cultivar from Italy, with a loading component of 0.85.**

Key words: Genetic diversity, *Cucurbita pepo*, cluster analysis, random amplified polymorphic DNA (RAPD).

INTRODUCTION

Cucurbita pepo is a worldwide cultivated fruit and economically important species of the genus *Cucurbita* L. It belongs to the family Cucurbitaceae, one plant group with the most species supply human with edible products and useful fibers (Bisognin, 2002). In the genus Cucurbita, five domesticated species were characterized, including *Cucurbita argyrosperma*, *Cucurbita ficifolia*, *Cucurbita maxima*, *Cucurbita moschata* and *C. pepo*, in addition to ten wild species. (Robinson and Decker-Walters, 1997). Most species originated in Mexico with only the species *C. maxima*, being native to South America.

C. pepo is the most diverse of these species and has traditionally comprised two subspecies; pepo and ovifera (Decker, 1988; Sanjur et al., 2002; Nesom, 2011). The former appears to be originated in Mexico while the second originated in the eastern half of United States. Each subspecies encompass several horticultural cultivar groups. *C. pepo* subspecies pepo L. includes pumkin, vegetable marrow, cocozelle and zucchini. *C. pepo* subspecies ovifera (syn. texana) includes acorn, scallop, croockneck and straightneck (Ferriol et al., 2003; Paris, 1986; Paris et al., 2003).

On the basis of archaeological records, *C. pepo* appears to be one of the first domesticated species. The oldest have been declared in the eastern of the United States (4000BC.) and in Mexico (8750BC).

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The records also reveal that the species may have been domesticated having Cucurbita fraterna and C. texana as possible progenitors. The species was introduced to Europe only about 500 years ago (Whitaker, 1947). Zucchini group is today the most widely grown and economically important group of *C. pepo* ssp. pepo. It is one of the easiest fruits to cultivate in temperate climate. It has a reputation for overwhelming production (Paris, 2010).

In a culinary context, the zucchini is treated as a vegetable, which means it is usually cooked and presented as a savory dish or accompaniment. However, botanically, the zucchini is an immature fruit, being the swollen ovary of the zucchini flower. Zucchini is considered on the list of the very low calories vegetables that contains no saturated fats and cholesterol. It is a moderate source of folates which is important in cell division and in DNA synthesis. It is a very good source of potassium, a vital intra-cellular electrolyte. Fresh fruits are rich in vitamin A, flavonoid, polyphenolic antioxidants such as carotenes, Lutein and zeaxanthin. These compounds help scavenge harmful reactive oxygen species ROS. It is also a good reservoir of antioxidant vitamin C, vitamin B-complex and mineral like iron, manganese, phosphorus and zinc (Achi et al., 2005).

Characterization based on morphological and horticultural traits have some disadvantages as being influenced by environmental factors (Dey, 1997; Sammour et al., 2007). It is tedious, unreliable and time-consuming. Instead, molecular characterization have proven many advantages, being able to distinguish polymorphisms which not produce phenotypic variation, highly polymorphic, easy and fast to detect and not possessing pleiotropic effects. Moreover, they can facilitate rapid screening of large numbers of genotypes for polymorphic loci (Williams et al., 1990).

Random amplified polymorphic DNA (RAPD) has established as a good genetic marker system to assay and evaluate the genetic diversity among species, among populations and furthermore among individuals in the same population (Ding et al., 2009; Mathew et al., 2010). It is an informative marker that screen large in the genome; either the expressed or the non-expressed and even the regulatory sequences (Rakhee et al., 2004; Elena et al., 2010).

Most studies proved RAPD marker reliability in estimating interspecific genetic relationships. The advantages of RAPD over other DNA-Based methods include a lack of the requirement for sequence information of the species, ease and speed of the assay, little amount of DNA required, no use of radioactivity and the ability to provide markers in genomic regions with repetitive DNA sequences (Dos-Santos et al., 1994; Hallden et al.1994; Thormann et al. 1994).

There are quite few studies concerning the molecular analysis of Cucurbita species. The RAPD markers were used to analyze the genetic diversity among *C. moschata* Lanraces from Korea, Southern Africa and other geographical origins (Youn and Chung, 1998; Baranek et al., 2000).

Ferriol et al. (2003) studied the genetic diversity among nineteen spanish accessions of C. maxima using two different molecular markers; sequence related ampli-fied polymorphism (SRAP and RAPD. More recently Ferriol et al. (2004a, b) employed the SRAP and RFLP molecular marker for analyzing the diversity among a large number of *C. Moschata* and *C. maxima* Landraces., Most of the studies that discuss the genetic variability within *C. pepo* using different molecular markers (RFLP, AFLP, RAPD, ISSRs) have been focused on the assess-ment of the genetic and evolutionary relationships between the wild and domesticated types, between the two subspecies or among the cultivars groups with only few representatives of landraces (Ferriol et al., 2003; Paris et al., 2003).

Therefore, the objectives of this study is to; First, discriminate and clarify the genetic relationships among the available seven *C. pepo* ssp. pepo cultivars using RAPD analysis as a basic requirement of further crop improvement. Second: to compare the natural variation present in a collection of the Egyptian landrace and the other commercial cultivars and hybrids to test the efficacy of RAPD for *C. pepo* cultivar identification.

MATERIALS AND METHODS

Plant material

Six cultivars and one Landrace belonging to *C. pepo* subspecies pepo (zucchini group) were donated from the Vegetables and Horticultural Research Center, The Agricultural Ministry). The represented Zucchini cultivars were Al-Escandrani landrace, D64-27, Amira, Mansoura, MHTSQ-05, MHTSQ-06 and Amjjed. The cultivars of this group were originated in different countries (Table 4).

Plant germination

Seeds were germinated at 25 to 30°C in the soil. Young fresh leaves were collected and stored in a -80°C deep freezer until use.

DNA extraction

DNA was extracted and purified from all samples using Qiagen DNeasy™ Plant Minikit following the protocol of the manufacturer (Qiagen Inc, Valencia, CA).

RAPD analysis

RAPD was performed as described by Williams et al. (1990) with minor modifications. Briefly, polymerase chain reaction (PCR) amplification was performed in 25 µL reaction mix containing 20.40 ng genomic DNA, 0.5 unit Taq polymerase (Sigma), 0.2 mM each of dATP, dCTP, dGTP, dTTP, 10 Pico mole random primer 5 µL amplification buffer. 1.5 µL of MgCl2 and 9.75 µL of distilled H2O. The reaction was assembled on ice, overlaid with a drop of mineral oil. Amplification was performed for 45 cycles using Biometera Uno

Table 1a. Name and sequences of the selected twenty random primers used in RAPD-PCR analysis. The highlighted primers are the primers resulted in polymorphism.

Primer name	Sequence (5'→3')
A-01	CAGGCCCTTC
A-02	TGCCGAGCTG
A-03	AGTCAGCCAC
A-04	AATCGGGCTG
B-01	GTTTCGCTCC
B-02	TGATCCCTGG
B-03	CATCCCCCTG
B-04	GGACTGGAGT
B-10	CTGCTGGGAC
B-14	TCCGCTCTGG
B-19	ACCCCGGAAG
G-01	CTACGGAGGA
G-02	GGCACTGAGG
G-03	GAGCCCTCCA
G-04	AGCGTGTCTG
J-11	ACTCCTGCGA
Z-01	TCTGTGCCAC
Z-02	CCTACGGGGA
Z-03	CAGCACCGCA
Z-04	AGGCTGTGCT

thermal cycler, as follows: One cycle at 95°C for 3 min and then 44 cycles at 92°C for 2 min, 37°C for 1 min and 72°C for 2 min. Reaction was finally incubated at 72°C for 10 min and further incubated on 4°C . Seven primers were selected for RAPD analysis based on their ability to amplify *C. pepo* genome and produce reproducible amplification patterns (Table 1).

The amplification products were separated by electrophoresis on 1.5% agarose in 50X TAE buffer (Tris-acetate EDTA buffer: 242 g Tris-base, 57.1 ml glacial acetic acid and 100 ml EDTA (0.5 M pH 8.0) stained with 0.2 µg/ml ethidium bromide and photographed under UV light. Sample was loaded by using 10 µl PCR-product and 2 µl loading buffer. 100 bp DNA ladder (Fermentas) was used.

Data analysis

The band identification was based on the mobility of DNA fragments by numerous side-by-side comparisons of DNA extracts. The genetic diversity among the accessions was evaluated by Jaccard similarity index, multivariate analysis (cluster and principal component analysis (PCA) analyses). The analyses were performed using the frequencies of scored bands calculated for the accessions. The dendrogram was constructed through the average linkage-joining rule, using the software package "SYSTAT for Windows", Version 7.0 copyright (C) 1997, SPSS INC. The RAPD data were analyzed using POPGENE version 1.31 Microsoft Window-based Freeware for Population Genetic Analysis.

RESULTS

Twenty (20) primers were used to elucidate the genetic diversity between seven *C. pepo* cultivars by amplifying the extracted DNA using RAPD-PCR analysis. The sequences of these primers are listed in Table 1a and b. The RAPD profile of the amplified products is shown in Figure 1. The number of bands and the degree of polymorphism revealed by each primer are given in Table 2. Totally, 87 consistent and differential amplification products were generated with seven (out of 20) decamer arbitrary primers. For each genotype, fragments were amplified per primer between 8 to 18 with an average of 13 bands/primer (Table 2). In general, the levels of polymorphism were varied with different primers among the different squash cultivars. The percentage of polymorphism produced by each primer differed from one primer to the other. Out of the total bands, 69 bands were polymorphic (Figure 1). The maximum value of polymorphism was 100% produced by the primers OPA-04 and OPZ-03. The minimum value of polymorphism was 27% produced by the primer OPB-02 with an average polymorphism of 70.9% across all the genotypes.

Table 1b. Name and sequences of the primers resulted in polymorphism using RAPD-PCR analysis.

Primer Name	Sequence (5'→3')
A-04	AATCGGGCTG
B-01	GTTTCGCTCC
B-02	TGATCCCTGG
B-04	GGACTGGAGT
G-02	GGCACTGAGG
Z-02	CCTACGGGGA
Z-03	CAGCACCGCA

The different cultivars could be distinguished either by fragments combination or by specific cultivar fragments. Twenty five (25) bands were unique amplification products and could be used as molecular marker to distinguish each cultivar. The RAPD profile is shown in Figure 1a, b, c and d. The bands generated by the primer OPB-04 with the sequence length of 391 and 351 bp were unique for D64-27 cultivar originated in Italy; while, the bands generated by the primer OPZ-02 with the sequence length of 716 and 360 bp were unique for Al-Escandrani landrace from Egypt. There was only one case, where a specific band, present in all other cultivars, was absent in a single cultivar (D64-27) (the band with sequence length of 140 bp and generated by the primer OPB-04).

The level of genetic diversity among RAPD fragments was calculated with Jaccard coefficient of similarity (Table 3). The overall mean similarity index calculated by Jaccard similarity index (JSI) for pair wise combination of the amplified fragments generated by the seven arbitrary primers on the genomic DNA of the seven cultivars of *C. pepo* ranged from 0.352 to 0.816 with an average of

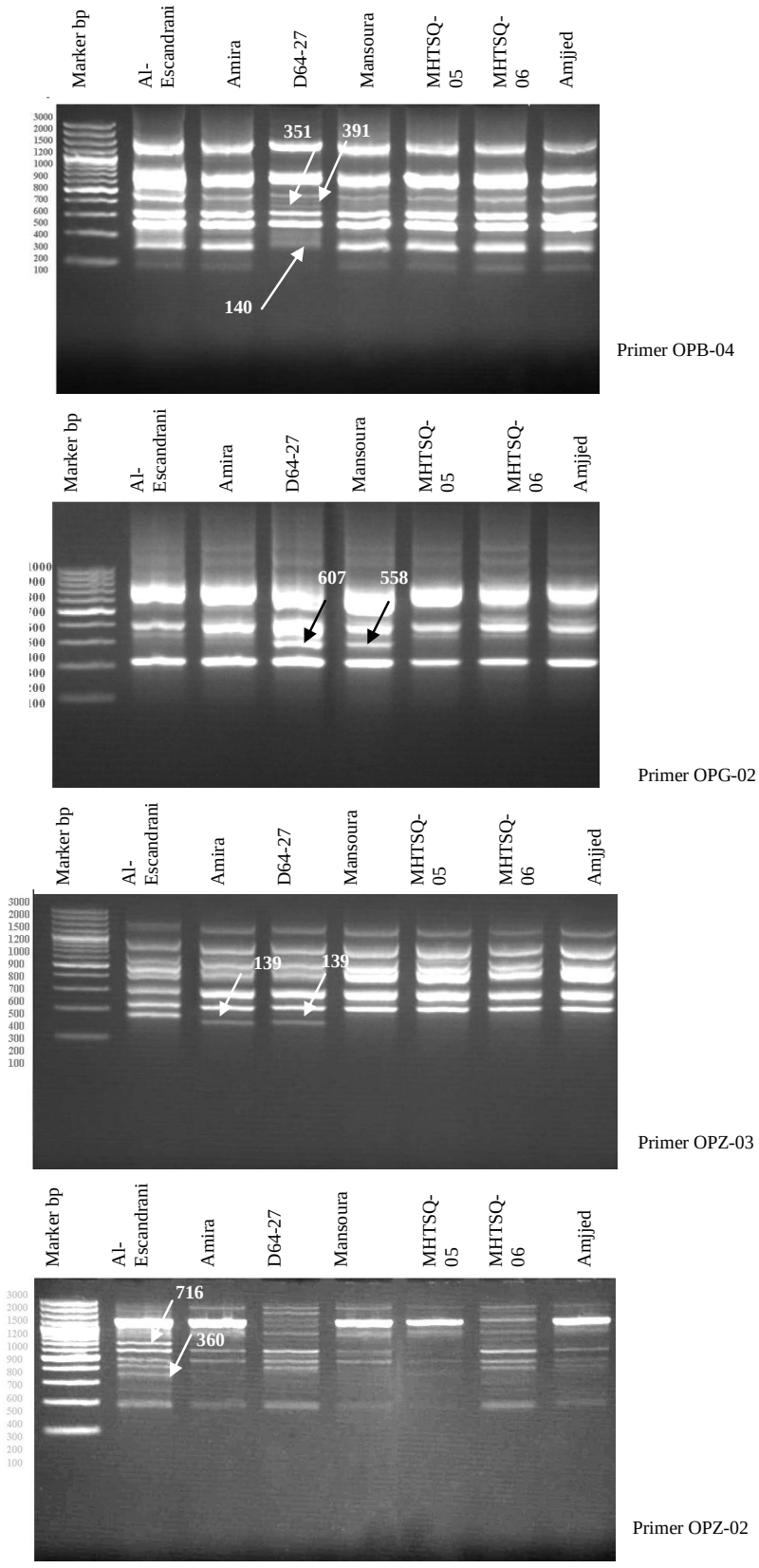

Figure 1. RAPD profiles illustrating bands amplification in the seven cultivars of *C. pepo* using four primers B04, G02, Z03 and Z02. DNA ladder Mix (100-3000 bp). Markers bands are indicated by arrow with molecular size.

Table 2. RAPD polymorphism among seven squash cultivars.

Unique product	Polymorphism (%)	Number of polymorphic bands	Total number of bands	Primer
6	100	13	13	OPA-04
-	38	3	8	OPB-01
1	27	3	11	OPB-02
2	64	7	11	OPB-04
6	88	14	16	OPZ-02
7	100	18	18	OPZ-03
3	79	11	14	OPG-02

Table 3. Jaccard Binary similarity coefficients between cultivars of *C. pepo*.

	Acc.1	Acc.2	Acc.3	Acc.4	Acc.5	Acc.6	Acc.7
Acc.1	1						
Acc.2	0.597	1					
Acc.3	0.486	0.678	1				
Acc.4	0.524	0.655	0.548	1			
Acc.5	0.469	0.618	0.469	0.792	1		
Acc.6	0.5	0.567	0.5	0.75	0.816	1	
Acc.7	0.371	0.444	0.352	0.6	0.654	0.717	1

0.682. The cultivars of *C. pepo* were divided into three groups: (1) the cultivar MHTSQ-06 and the cultivar MHTSQ-05 with the highest index of similarity that equal 0.816. Both cultivars were H1 hybrid result from cross between Al-Escandrani landrace as one parent and an American inbred line as the other parent; (2) the cultivar Amjjed originated in USA with the cultivar MHTSQ-06, the cultivar MHTSQ-06 with the cultivar Mansoura originated in Italy and the cultivar MHTSQ-05 with the cultivar Mansoura with a high mean indices of similarity equal 0.717, 0.750 and 0.792, respectively; (3) The lowest Jaccard similarity index was recorded between the cultivar Amjjed from USA with both the landrace Al-Escandrani from Egypt and the cultivar D64-27 from France with the mean similarity index of 0.371 and 0. 352, respectively.

Genetic diversity among the seven cultivars of *C. pepo* was investigated by cluster analysis using Euclidean distance matrix on average linkage. The phenogram constructed using each cultivar as an operational taxonomic unit (OUT) and including all the DNA fragments generated by the seven primers (Figure 2). The cluster analysis showed two main groups. G1 group contained the cultivar Al-Escandrani (the Egyptian Landrace) and G2 group contained the rest cultivars. G2 group is subdivided into two subgroups (G2a and G2b).

G2a included the two cultivars Amira and D64-27. Both cultivars originated in France. G2b separated the rest cultivars to two clusters. The first cluster included the cultivar Amjjed from USA solitary. The second cluster gathered two H1 hybrids (Al-Escandrani landrace x USA inbred line) with a third cultivar named as Mansoura and originated in Italy.

The principal component analysis (PCA) is one of the multivariate approaches to grouping based on the similarity coefficients of variance – covariance value of the component traits of the entities. The matrix of eigenvectors and values of the principle components PCs resulting from the interaction of the RAPD data (Table 4), indicated that all the DNA fragments generated by the seven primers on the DNA of the seven cultivars of *C. pepo* influencing 71.47% of the variability accumulated up to the first two components of PCA. The first component explained 51.24% of the total variation. The second component showed 20.23%. All the studied cultivars were separated on the first principal component.

The factorial plot showed all the cultivars aggregated in one direction. In parallel with the cluster tree, the cultivar MHTSQ-06, the cultivar MHTSQ-05 and the cultivar Mansoura have the same loading component of about 0.85, though they aggregated near to each other on the plot. Also the cultivar Amira and the cultivar D64-27 were aggregated together with approximately the same loading component of about 0.7.

DISCUSSION

The knowledge of genetic diversity of a crop is essential for the parental selection, which maximizes the genetic

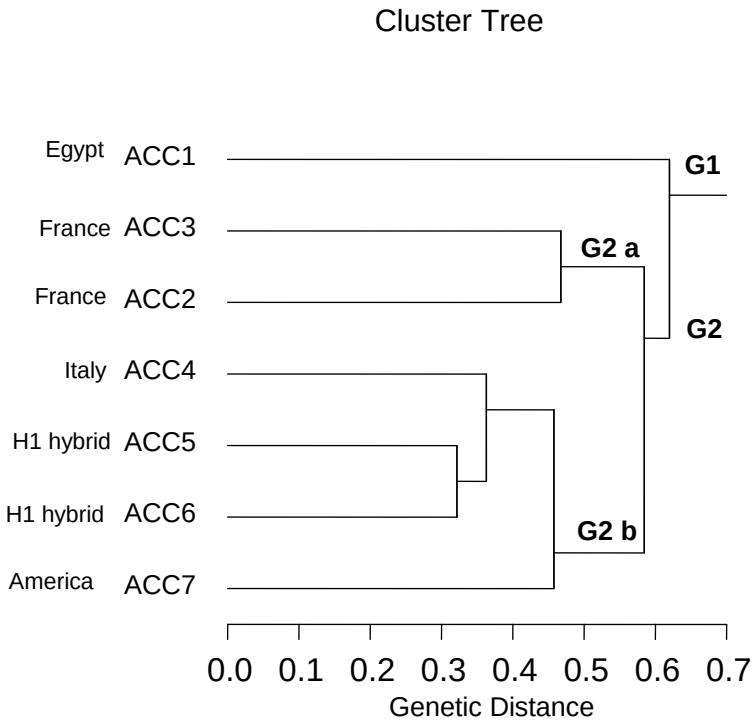

Figure 2. Dendrogram showing the genetic relationships among the seven cultivars of *C. pepo* based on genetic distance.

Table 4. Name, Origin, matrix of eigenvectors and values of the principle components for RAPD data of *C. pepo* cultivars.

Acc. Number	Name	Origin	Principal component 1	Principal component 2
1	Al-Escandrani	Egypt	0.4	-0.469
2	Amira	France	0.714	-0.506
3	D64-27	France	0.704	-0.649
4	Mansoura	Italy	0.886	0.008
5	MHTSQ-05	H1 hybrid	0.892	0.204
6	MHTSQ-06	H1 hybrid	0.862	0.29
7	Amjjed	USA	0.632	0.627
	Variance explained by components		3.587	1.417
	Percent of total variance explained		51.242	20.237
	Accumulated eigenvectors		51.242	71.48

improvement. Though, identification and utilization of the diverse germplasm is a core issue for plant breeding. The accurate description of different genotypes and the patterns of the genetic diversity help determining future breeding strategies and facilitate introgression of diverse germplasm into the current commercial squash genetic base. Furthermore, the estimated genetic diversity of these cultivars increases the use and interest of data already existing in the gene banks.

Our present study investigates a high intraspecific genetic diversity among the studied *C. pepo* cultivars. The variation assessed by 87 RAPD polymorphic bands generated by seven primers in the represented germplasm. The DNA fragments generated by RAPD primers were different in number, intensity and position indicating high genetic variation between the studied cultivars. The average polymorphism was 70.9% among the *C. pepo* cultivars which confirmed the high genetic variation between them. The primers OPB-01 and OPB-02 were characterized with the lowest number of polymorphic bands (three bands); whereas, the primers OPA-04 and OPZ-03 were characterized by the highest

number of generated polymorphic bands (13 and 18 bands, respectively). The variation between reproducible bands generated by each primer depends on primer, sequence and the extent of variation in specific genotype (Chan and Sun, 1997; Shiran et al., 2007; Shukla et al., 2006).

With RAPD technique, some fragments were uniquely amplified in single cultivar such as the bands with the sequence length of 391 and 351 bp in the D64-27 cultivar from France and the bands with the sequence length of 716 and 360 bp in the Egyptian landrace El- Escandrani. In fact, these fragments are of great interest in optimal management of germplasm collections, as they facilitate the identification of cultivars and duplicates and verify possible pollen or seed contamination during conservation activities (Ferriol et al., 2004 a,b).

The output of Jaccard binary similarity coefficient and cluster analysis based on all DNA fragments generated by the seven primers showed the strongest homogeneity between the MHTSQ-05 cultivar and MHTSQ-06 cultivar with mean similarity index of 0.816. Both hybrids resulted from cross between El-Escandrani landrace x American inbred lines. A strong homogeneity was also found between Mansoura cultivar from Italy with either the MHTSQ-05 and MHTSQ-06 cultivars with mean similarity indices of 0.792 and 0.750, respectively. This homogeneity might be attributed to that these cultivars originating from one progenitor; Amjjed cultivar from USA.

The cluster analysis based on the comparison in the similarity matrix between the RAPD fragments generated by the seven primers resulted in more reliable data. From the dendrogram, we can predict that there was a strong relation between the genetic diversity and the geographical origins; whereas, the cultivars from different geographical locations were often unique and tend to be clustered in one part of the dendrogram. This result clearly suggests that the variation analyzed is determined not only by genetic factors but also by environmental differences. This agreed with Naghavi and Jahansouz (2005).

The constructed phenogram exhibited that all the genetic distance among the examined cultivars was ~59%. The Egyptian landrace El-Escandrani clustered independently from all the other studied cultivars. This finding indicated its unique banding pattern over the rest cultivars. The results ensured the wide genetic base of landraces, as they are characterized by a specific adaptation to the environmental conditions of the area of cultivation. Munazza et al. (2009) reported that the assessment of genetic diversity within and between landraces should have priority for variety improvement. Therefore, our results recommended the Egyptian zucchini landrace that have a level of diversity higher than that of the commercial cultivars and hybrids. So, it can be considered as a reservoir of alleles useful for breeding; because divergent genotypes may have a good breeding value (Gwanama et al., 2000).

The dendrogram also separated the cultivars originated from Europe and USA together with the hybrids that have one parent from USA; all in one major cluster. The Amira cultivar and the D64-27 cultivar were separated in distinct group. Both originated in France. This finding ascertained the fact that the distribution of the cultivars here is dependent on the geographical location. This agreed with Gwanama et al. (2000), who studied the genetic relationships among accessions of C. moschata in Zambia and Malawi. He observed that the accessions were grouped according to criteria of the geographic origin and the degree of breeding. The two hybrids MHTSQ-05 and MHTSQ-06 resulted from cross between El-Escandrani landrace x American inbred lines were separated in a second distinct group at a genetic distance of 30%. In general, a lower genetic variability is found within commercial hybrids compared with commercial cultivars and landraces, which is consistent with the narrower origin of hybrids and genetic erosion due to intensive breeding (Formisano et al., 2010). Moreover, both MHTSQ-05 and MHTSQ-06 cultivars were located together with Mansoura cultivar originated in Italy in one cluster. All these cultivars had Amjjed cultivar from USA as a possible ancestor. This finding ascertains the fact that Central and North America are the primary centers of diversity. It may also indicate the great diversification that took place in this species after the first *C. pepo* fruits and seeds arrived in Europe from America (Ferriol et al., 2003).

It is notably on the phenogram that Mansoura cultivar from Italy was located between the two cultivars originated in France on one side and the two MHTSQ hybrids on the other side. This result may be due to the fact that, since the 16th century, various squash cultivars migrate from different parts of South America where the primary center of diversity to reach Europe (Ferriol et al., 2004 a,b). It also may be due to the geographical location of Italy nearby France. Therefore, spreading new types selected in the two continents both ways through migration, hybridization and introgression. Meanwhile, *C. pepo* species is an out crossing and open-pollinated species. This character generates a great diversity of phenotypes; most of them being intermediate forms (Ferriol et al., 2003). It also suggested that the Italian *C. pepo* cultivars did not originate from squashes coming from a single American origin.

On the dendrogram, the Amjjed cultivar originated in USA was located far from the Egyptian landrace El-Escandrani; with a low similarity index of 0.371. This finding may due to the different geographical origins. However, both can be considered a vital genetic resource as parent for further breeding programs. Actually, the maximum variability for selection in segregating populations may be achieved by utilizing genotypes from different cluster as parents for crosses (Gwanama et al., 2000). The multivariate methods of analysis such as PCA

used in the present study provided an effective way of evaluating germplasm material in order to identify materials that could be further evaluated or utilized.

In our results, the principal component analysis for the studied cultivars based on all DNA fragments generated by seven primers shows that the first two components accounted for 71.47 of the total variance of all cultivars. The separation of almost all cultivars was on the first component. This result indicates a high degree of correlation among the studied cultivars. The Egyptian landrace El-Escandrani had the lowest loading component of 0.400 indicating its low correlation with the other cultivars as it is selected for the Egyptian growing conditions. The PCA also confirmed the strong correlation between the two MHTSQ hybrids with Mansoura cultivar from Italy. This result was clarified by the similar high loading component of about 0.85.

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Full Length Research Paper

Genetic variation of twenty autosomal STR loci and evaluate the importance of these loci for forensic genetic purposes

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The aim of this study was of twofold. One was to determine the genetic structure of Iraq population and the second objective of the study was to evaluate the importance of these loci for forensic genetic purposes. FTA® Technology (FTA™ paper DNA extraction) utilized to extract DNA. Twenty (20) STR loci and Amelogenin), including D3S1358, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, THO1, vWA, D21S11, D7S820, TPOX, D8S1179, FGA, D2S1338, D5S818, D6S1043, D12S391, D19S433 and Amelogenin amplified by using power plex21® kit. Polymerase chain reaction (PCR) products detected by genetic analyzer 3730xL then data analyzed by PowerStatsV1.2. Based on the allelic frequencies, several statistical parameters of genetic and forensic efficiency have been estimated. This includes the homozygosity and heterozygosity, effective number of alleles (n), the polymorphism information content (PIC), the power of discrimination (DP) and the power of exclusion (PE). The power of discrimination values for all tested loci was from 75 to 96%; therefore, those loci can be safely used to establish a DNA-based database for Iraq population. The high PIC values of the selected markers confirm their usefulness for genetic polymorphism studies and linkage mapping programs in human as well. The mean heterozygosity observed, is expected to have mean PIC values across the 20 loci which were 0.77, 0.81 and 0.78, respectively, indicating high gene diversity.

Key words: Autosomal STR, genetic variation, Iraq, statistical parameters.

INTRODUCTION

Microsatellites refer to DNA with varying numbers of short tandem repeats (Klintschar et al., 2006) between a unique sequence. DNA regions with repeat units that are 2 bp to 7 bp in length or most generally short tandem repeats (STRs) or simple sequence repeats (SSRs) are generally known as microsatellites (Ellegren, 2004).

In the core repeated bases, long repeat units may contain several hundred to thousands (Butler and Hill, 2012). Within the DNA there are length and sequence

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Abbreviations: STRs, Short tandem repeats; **SSRs,** simple sequence repeats; **EDTA,** ethylene diamine tetraacetic acid; **TE,** tris-EDTA buffer; **PCR,** polymerase chain reaction; **Ho,** observed heterozygosity; **He,** expected heterozygosity; **PD,** power of discrimination; **PE,** probability or power of exclusion; **PIC,** polymorphism information content; **HWE,** Hardy Weinberg equilibrium; **RFLP,** restriction fragment length polymorphism; **CDP,** combined discrimination power; **PI,** paternity index; **RMP,** random match probability.

polymorphisms (Silvia et al., 2009). DNA can be used to study human evolution using human genome analysis regions that are not subjected to selection pressure (Mats et al., 2007; Imad et al., 2014). Besides, information from DNA typing provides vital information in medico-legal with polymorphisms allowing for more biological studies (Walkinshaw et al., 1996).

It has been found that microsatellites are evenly distributed in the genome on all chromosomes and all regions of the chromosome (Ensenberger et al., 2010; Imad et al., 2014). They can also be found inside gene coding regions, introns, and in the non-gene sequences. Most microsatellite loci are really small, ranging from a few to a few hundred repeats and this small size of microsatellite loci is important for PCR-facilitated genotyping. Basically, microsatellites containing a higher number of repeats are more polymorphic.

The Cooperative Human Linkage Center http://www.chlc.org evaluates the genetic markers and the loci are selected from there (Table 1) which provides particulars on the additional STR loci, chromosomal location and repeat sequence for each core STR locus (Ruitberg et al., 2001; Klintschar et al., 2004; Klintschar et al., 2005). Therefore, the repeat motif for each STR marker is listed based on this. A significant fact is that STR allele sizes are measured relative to an internal size standard during electrophoresis. This depends on the DNA strand that is labeled using a dye that may have a different apparent measured size.

The PowerPlex® 21 System is compatible with automated PCR instrument and with the ABI PRISM® 3100, 3100-Avant, 3130, 3130xl, 3500 and 3500xL Applied Biosystems Genetic Analyzers. In the United States, Europe and Asia the PowerPlex® 21 System is used and it increases the discriminatory power and data-sharing possibilities by incorporating informative loci. The PowerPlex® 21 System includes the 13 CODIS core STR loci, two loci commonly used in Europe (D1S1656 and D12S391).

In China, the D6S1043 locus is commonly used. (Amelogenin, Penta D, Penta E, D2S1338 and D19S433) are several additional markers used throughout the world. In forensic casework and DNA databases, addition of new autosomal is very important to increase the discrimination power for human forensic identification.

This study was aimed at investigating the genetic variation and Forensic efficiency parameters of 20 autosomal STR loci from random unrelated individuals in the middle and south of Iraq.

MATERIALS AND METHODS

Population

Four hundred (400) healthy, randomly chosen individuals deriving from the middle and south of Iraq provinces (Baghdad, Babil, Diwania and Basrah). The number and ethnicity of individuals were chosen in order to obtain a population sample to achieve the highest possible representation of the major ethno-religious and tribal groups of the country living in these central and southern areas.

DNA extraction

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 (Dobbs et al., 2002). 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 three times in 100 µl Whatman FTA purification reagent. Each was washed and 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.

PCR

Amplifications of 20 STR loci D3S1358, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, THO1, vWA, D21S11, D7S820, TPOX, D8S1179, FGA, D1S1656, D5S818, D6S1043, D12S391, D19S433 and Amelogenin were performed by using the PowerPlex®21 kit. The PCR program was follow as: 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 4°C was soak. Keep the amplified samples at -20°C in a light-protected box.

Typing

Using the ABI Prism1 3130xl 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® V3.2 software (Applied Biosystems, Foster City, CA, USA). By comparison, the size of a sample's alleles to size the alleles in allelic ladders for the same loci are being tested in the sample, the STR genotyping was conducted.

Quality control

Experimental procedures were performed according to the guidelines of the external blind proficiency test of the GEDNAP (http://www.gednap.org) (Rand et al., 2002; Rand et al., 2004).

Statistical data analysis

The PowerStatsV1.2 (Promega, Madison, USA) was used to calculate the observed heterozygosity (Ho), power of discrimination (PD), probability of exclusion (PE), and polymorphism information content (PIC). Arlequin software program (Schneider et al., 1998) was used to conduct the exact test of population differentiation. In addition, Arlequin software program was used for the expected heterozygosity (He), Hardy Weinberg Equilibrium (HWE) and linkage equilibrium tests as well as for the F-statistics. Where test results with P-values less than 0.05 were observed, and the Bonferroni correction had to be applied to the data. The Bonferroni procedure (Weir, 1996) adjusted the rejection level for the smallest P-value at an overall level of $\alpha = 5\ \%$ to $0.05/x$, where x is equal to the number of tests conducted on the data. The Ho and He values were calculated by means of the same software program.

Table 1. Information on 21 autosomal STR loci present in The PowerPlex® 21 System kits Adapted from (Cotton et al., 2000; Wiegand et al., 1993) physical positions are from (Schneider et al., 1998).

STR Locus[a] [n]	Label	Physical position	Chromosomal Location1	Repeat Sequence 5´ to 3´
Amelogenin	Fluorescein	X and Y	Xp22.1-22.3 and Y	NA
D3S1358	Fluorescein	Chr 3 (45.582 Mb)	3p21.31	TCTA Complex
D1S1656	**Fluorescein**	Chr 1 (230.905 Mb)	1q42	TAGA Complex
D6S1043	**Fluorescein**	**Chr 6 (92.450 Mb)**	**6q15**	**AGAT**
D13S317	Fluorescein	**Chr 13 (82.692 Mb)**	**13q31.1**	**TATC**
Penta E	**Fluorescein**	Chr 15 (97.374 Mb)	15q26.2	AAAGA
D16S539	JOE	**Chr.16(86.386Mb)**	**16q24.1**	**GATA**
D18S51	JOE	Chr 18 (60.949 Mb)	18q21.33	AGAA
D2S1338	JOE	Chr 2 (218.879 Mb)	2q35	TGCC/TTCC
CSF1PO	JOE	Chr 5 (149.455 Mb)	5q33.1	AGAT
Penta D	**JOE**	Chr 21 (45.056 Mb)	21q22.3	AAAGA
TH01	TMR-ET	Chr 11 (2.192 Mb)	11p15.5	AATG (19)
vWA	TMR-ET	**Chr12(6.093 Mb)**	**12p13.31**	**TCTA Complex (19)**
D21S11	TMR-ET	Chr 21 (20.554 Mb)	21q21.1	TCTA Complex (19)
D7S820	TMR-ET	Chr 7 (83.789 Mb)	7q21.11	GATA
D5S818	TMR-ET	Chr 5 (123.111 Mb)	5q23.2	AGAT
TPOX	CXR-ET	Chr 2 (1.493 Mb)	2p25.3	AATG
D8S1179	CXR-ET	Chr 8 (125.907 Mb)	8q24.13 (125.976Mb)	TCTA Complex (19)
D12S391	**CXR-ET**	Chr 12 (12.450 Mb)	**12p12(12.341Mb)**	AGAT/AGAC **Complex**
D19S433	CXR-ET	**Chr19(30.416 Mb)**	(35.109Mb)	AAGG Complex
FGA	CXR-ET	Chr 4 (155.509 Mb)	4q28 (155.866Mb)	4q28 (155.866Mb)

a database of sequence-tagged sites (STSs) available on the NCBI website: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unists.
n The 13 CODIS core loci are highlighted in bold font.

RESULTS AND DISCUSSION

Allele frequency of common autosomal genetic loci

After the samples have been collected, DNA extracted and PCR amplified were genotyped for the 20 STR loci of interest. The genotyping information was then converted into allele frequencies by counting the number of times each allele was observed. Allele frequencies for each of the 20 STR loci in the Iraq population sample are shown in (Tables 2 and 3).

Since there are some alleles which were not sampled sufficiently and an estimate of an allele frequency is uncertain if the allele is so rare that it can represented only once or a few times in a dataset, it is recommended that each allele was observed at least five times to be used in forensic calculations (Butler, 2007). The minimum allele frequency is 5/ (2n) where n is the number of individuals sampled and 2n is the number of chromosomes (as autosomes are in pairs due to inheritance of one chromosome from each parent).

In the loci D5S818 (allele 13) the highest allele frequencies are found and the lowest allele frequencies are at allele 13 as seen in D3S1358 locus. D21S11 and D18S51 loci illustrate the largest number of different alleles. The following locations of the most common alleles at the 20 loci were allele 13 for (D5S818, D8S1179 and D18S51), allele 10 for (D7S820 and Penta D), allele 16 for (D2S1338 and D2S1338), allele 11 for (TPOX, D16S539, Penta E, CSF1PO and D6S1043) loci, allele 12 for (D13S317 and D1S1656), allele 30.2 for D21S11 locus, allele 9 for THO1 locus, allele 13.2 for D21S11 locus, allele 6 for THO locus, alleles 17.3 and 16 for VWA locus, allele 30.2 for D19S433 locus, allele 17 for D3S1358 locus, allele 18 for D5S818 locus, allele 18 for D12S391 locus, allele 14.2 for D12S391 locus and allele 25 for FGA locus.

The best indicators of the genetic polymorphism within the sample are verified by the number of alleles and the expected heterozygosity is found in the Iraq population. Basically, the number of alleles is highly associated with the size of the sample. This is due to the presence of unique alleles in populations, which occur in low frequencies. The usefulness of the markers for genetic screening is verified by the number of alleles scored for each marker.

The number of alleles and the expected heterozygosities detected in Iraq population are good indicators of the genetic polymorphism within the breed. Generally, the number of alleles is highly dependent on the sample

Table 2. Allele frequencies and forensic efficiency parameters (D8S1179- D3S1358) genetic loci.

Allele	D8S1179	D7S820	D21S11	THO1	vWA	TPOX	D13S317	D5S818	D12S391	D3S1358
2.2	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-
6	-	-	-	0.0455	-	-	-	-	-	-
7	-	0.0265	-	0.1713	-	-	-	-	-	-
8	0.127	0.1896	-	0.1326	-	0.0197	0.2318	-	-	-
9	0.0064	0.1003	-	0.2362	-	0.0383	0.0373	0.0228	-	-
9.3	-	-	-	0.1344	-	-	-	-	-	-
10	0.076	0.3304	-	0.18	-	0.1013	0.0546	0.0124	-	-
10.2	-	-	-	-	-	-	-	-	-	-
11	0.1344	0.2054	-	-	-	0.2637	0.2248	0.0382	-	-
12	0.1153	0.1115	-	-	-	0.2005	0.343	0.0133	-	-
12.2	-	-	-	-	-	-	-	-	-	-
13	0.2153	0.0363	-	-	-	0.1985	0.0534	0.4375	-	0.0037
13.2	-	-	-	-	-	-	-	-	-	-
14	0.142	-	-	-	0.0917	0.178	0.0551	0.3588	-	0.04
14.2	-	-	-	-	-	-	-	-	-	-
15	0.0549	-	-	-	0.0594	-	-	0.0208	-	0.2267
15.2	-	-	-	-	-	-	-	-	-	-
15.3	-	-	-	-	-	-	-	-	-	-
16	0.0181	-	-	-	0.2824	-	-	0.0407	-	0.2394
16.2	-	-	-	-	-	-	-	-	-	-
16.3	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	0.2806	-	-	-	0.0795	0.3471
18	-	-	-	-	0.0588	-	-	-	0.3597	0.1231
19	-	-	-	-	0.0212	-	-	-	0.1827	0.02
20	-	-	-	-	0.2059	-	-	-	0.1783	-
20.2	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	0.0194	-
21.2	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	0.0615	-
22.2	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	0.042	-
23.2	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	0.0771	-
24.2	-	-	-	-	-	-	-	-	-	-
28	-	-	0.0614	-	-	-	-	-	-	-
28.2	-	-	-	-	-	-	-	-	-	-
29	-	-	0.1817	-	-	-	-	-	-	-
29.2	-	-	-	-	-	-	-	-	-	-
30	-	-	0.1393	-	-	-	-	-	-	-
30.2	-	-	0.1615	-	-	-	-	-	-	-
31	-	-	0.0596	-	-	-	-	-	-	-
31.2	-	-	0.0113	-	-	-	-	-	-	-
32	-	-	0.0485	-	-	-	-	-	-	-
32.2	-	-	0.0589	-	-	-	-	-	-	-
33	-	-	0.0589	-	-	-	-	-	-	-
33.2	-	-	0.0983	-	-	-	-	-	-	-
34	-	-	0.0029	-	-	-	-	-	-	-
34.2	-	-	0.1066	-	-	-	-	-	-	-
35	-	-	0.0112	-	-	-	-	-	-	-

Table 3. Allele frequencies and forensic efficiency parameters (Penta D - CSF1PO).

Allele	Penta D	D19S433	D2S1338	D18S51	D16S539	Penta E	FGA	D6S1043	D1S1656	CSF1PO
2.2	0.0186	-	-	-	-		-	-	-	-
5	-	-	-	-	-	0.0183	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-
7	0.0383	0.0023	-	-	-	0.0412	-	-	-	-
8	0.0217	-	-	-	0.1788	0.1988	-	-	-	0.0158
9	0.1614	0.0939	-	-	0.1012	0.0417	-	-	-	0.036
9.3	-	-	-	-	-	-	-	-	-	-
10	0.3985	0.0082	-	0.0086	0.2185	0.0987	-	0.0188	-	0.2636
10.2	-	-	-	0.0208		-	-		-	-
11	0.1015	0.1834	-	0.0454	0.251	0.2415	-	0.3412	0.1388	0.2858
12	0.0587	0.157	-	0.1782	0.0987	0.1015	-	0.2385	0.2387	0.2633
12.2	-	0.1148	-	-	-	-	-	-	-	-
13	0.1016	0.0726	-	0.2459	0.1003	0.0212	-	0.1215	0.0985	0.0622
13.2	-	0.2169	-	-	-	-	-	-	-	
14	0.0413	0.0745	-	0.17	0.0515	0.0088	-	0.0783	0.1615	0.011
14.2	-	0.0424	-		-	-	-	-	-	-
15	0.0186	0.0109	0.0583	0.1044	-	0.0298	-	0.0217	0.0983	0.0633
15.2	-	0.0598	-	-	-	-	-	-	-	-
15.3	-	-	-	-	-	-	-	-	0.0417	-
16	0.0398	-	0.2015	0.1471	-	-	-	0.0587	-	-
16.2	-	-	-	-	-	-	-	-	0.2012	-
16.3	-	-	-	-	-	-	-	-	-	-
17	-	-	0.1383	0.0473	-	-	-	0.0813	0.0213	-
18	-	-	0.0785	0.0111	-	-	-	0.0898	-	-
19	-	-	0.1513	0.0064	-	-	-	0.0622	-	-
20	-	-	0.0587	0.0112	-	-	0.1014	0.112	-	-
20.2	-	-	-	-	-	-	-	-	-	-
21	-	-	0.0388	0.0112	-	-	0.0379	-	-	-
21.2	-	-	-	-	-	-	-	-	-	-
22	-	-	0.0612	-	-	-	0.1817	-	-	-
22.2	-	-	-	-	-	-	0.1389	-	-	-
23	-	-	0.0083	-	-	-	0.0787	-	-	-
23.2	-	-	-	-	-	-	-	-	-	-
24	-	-	0.0717	-	-	-	0.1373	-	-	-
24.2	-	-	-	-	-	-	-	-	-	-
25	-	-	0.0617	-	-	-	0.2429	-	-	-
26	-	-	0.0717	-	-	-	0.0812	-	-	-
27	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-
28.2	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-
29.2	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-
30.2	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	-	-
31.2	-	-	-	-	-	-	-	-	-	-
32	-	-	-	-	-	-	-	-	-	-
32.2	-	-	-	-	-	-	-	-	-	-
33	-	-	-	-	-	-	-	-	-	-

the sample size because of the presence of unique alleles in populations, which occur in low frequencies and also because the number of observed alleles tends to increase with increases in population size. The number of alleles scored for each marker is an invaluable indicator of the future usefulness of the marker for genetic screening.

Finding the same number of alleles for certain different loci in various populations (e.g., Iran, Syrian, Emirates, Qatar and Egyptian populations) may indicate common ancestries (Reyhaneh et al., 2009; Alshamali et al., 2003; Ana et al., 2006; Clotilde et al., 2007). The frequency and the number of alleles, however, may be an indication for the degree of inbreeding within each population and thus reflects the homogeneity of the population. The analysis of STR polymorphisms by PCR-based method offers certain advantages over RFLP typing.

In recent years, short tandem repeat (STR) systems have gained importance in forensic analysis of biological specimens as well as in paternity testing, as an alternative to the use of restriction fragment length polymorphism (RFLP) analysis (Edwards et al., 1991; Hammond et al., 1994; Nakamura et al., 1987). The analysis of STR polymorphisms by PCR-based method offers certain advantages over RFLP typing: (1) STR loci can be typed with a high degree of specificity and sensitivity in a short time period, (2) these loci can be successfully amplified from a limited amount of DNA even if it is degraded, and (3) typing of multiple loci can be accomplished in a single multiplex reaction (Hochmeister et al., 1991; Lins et al., 1996; Mohammad and Imad, 2013a, b).

The amelogenin locus

The amelogenin locus occurs on both the X and Y chromosome and enables sex typing (Sullivan et al., 1993) was also located within the reference human genome sequence. AMELX is located on the X chromosome at 10.676 Mb. AMELY is located on the Y chromosome at 6.441 Mb. Amplification of Amelogenin generates different length products from the X and Y-chromosomes.

Observed heterozygosity and expected heterozygosity

A higher heterozygosity means that more allele diversity exists and therefore there is less chance of a random sample matching. Observed heterozygosity and expected heterozygosity all over the 20 loci are presented in (Table 4), and the observed heterozygosity oscillated between studied populations as illustrated in (Table 5). The observed heterozygosity in a population relies on the number and the frequency of alleles of each locus. Moreover, the distribution of genotypes in a population sample may deviate from HWE expectation in a number of ways. These include the presence of an excess of homozygotes (and a corresponding lack of heterozygotes) or an excess (or deficiency) of one or more classes of heterozygotes or a combination of those states. There are populations with low heterozygosity, lower than 65% in most tested loci.

Paternity index

The potential of a randomly selected man to pass the obligate gene is determined by using a database, which lists the frequency distribution of individual alleles within a given genetic system. Combined paternity index is an odds ratio that indicates how many times more likely it is that the alleged father is the biological father than a randomly selected unrelated man of similar ethnic background. The paternity index was high for all STR analyzed it ranged from 2.651 (TPOX) to 2.864 (D21S11).

Random match probability

The match probability is the probability for a random match between two unrelated individuals drawn from the same population. It is the sum of the frequency squared of each genotype; it ranged from 0.011 to 0.168.

Power of discrimination

Values for all tested loci were 75% for TPOX. Ranged from 80 to 89% for the D3S1358, D13S317, D5S818, D12S391, vWA, Penta D, D16S539, D1S1656 and CSF1PO loci, and ranged from 91 to 96%, for the rest of the loci. This infers that a DNA-based database for Iraq population can be safely used by using these loci. The highest PD observed in some populations is presented in (Table 6). The Penta E and Penta D loci included in the PowerPlex®21 PCR amplification kits were not typed in the Turkey, Emirates, Iran or Qatari populations because they used different kits in their genotyping studies. The Combined Discrimination Power (CDP) for the Iraq population of middle and south of Iraq for the corresponding 20 STR loci used has been calculated as 0.999999972. These results mean that those loci can be safely used to establish a DNA-based database for Iraq population.

Chance of exclusion

The Power of Exclusion (PE) can be calculated to express how rare it would be to find a random man who could not be excluded as the biological father of the child (Fisher, 1951; Chakraborty and Stivers 1996; Butler,

Table 4. Forensic efficiency parameters of the autosomal 21.

Locus	Observed heterozygosity (Ho)	Expected heterozygosity (He)	paternity index (PI)	Random match probability (RMP)	Power of discrimination (PD)	Chance of exclusion (CE)	polymorphic information content (PIC)	P-value*
S1179	0.784	0.829	2.807	0.012	0.912	0.528	0.802	0.423
D7S820	0.821	0.866	2.844	0.025	0.949	0.569	0.839	0.442
D21S11	0.841	0.886	2.864	0.045	0.969	0.585	0.859	0.48
THO1	0.785	0.83	2.808	0.011	0.913	0.529	0.803	0.424
vWA	0.755	0.8	2.778	0.041	0.883	0.499	0.773	0.394
TPOX	0.628	0.673	2.651	0.168	0.756	0.372	0.646	0.267
D13S317	0.727	0.772	2.75	0.069	0.855	0.471	0.745	0.366
D5S818	0.735	0.78	2.758	0.061	0.863	0.479	0.753	0.374
D12S391	0.72	0.765	2.743	0.076	0.848	0.464	0.738	0.359
D3S1358	0.766	0.811	2.789	0.03	0.894	0.51	0.784	0.405
Penta D	0.751	0.796	2.774	0.045	0.879	0.495	0.769	0.39
D19S433	0.803	0.848	2.826	0.007	0.931	0.549	0.821	0.442
D2S1338	0.832	0.877	2.855	0.036	0.96	0.576	0.85	0.471
D18S51	0.831	0.876	2.854	0.035	0.959	0.575	0.849	0.47
D16S539	0.766	0.811	2.789	0.03	0.894	0.51	0.784	0.405
PentaE	0.807	0.852	2.83	0.011	0.935	0.551	0.825	0.446
FGA	0.823	0.868	2.846	0.027	0.951	0.567	0.841	0.462
D6S1043	0.81	0.855	2.833	0.014	0.938	0.554	0.828	0.449
D1S1656	0.746	0.791	2.769	0.05	0.874	0.49	0.764	0.385
CSF1PO	0.715	0.76	2.738	0.081	0.843	0.459	0.733	0.354

Table 5. The observed heterozygosity in different populations.

Locus	Iraq	Turkey	Iran	Syria	Emirates	Qatar	Egypt	Gaza
D21S11	0.841	-	-	0.664	-	-	0.682	0.682
TPOX	0.628	0.567	-	-	-	-	-	-
Penta E	-	-	-	0.897	-	-	-	-
FGA	-	-	-	-	-	-	0.890	
D18S51	-	0.790	-	-	0.130	-	-	0.912
D2S1338	-	-	-	-	-	0.839	-	-
vWA	-	-	-	-	-	0.542	-	-
LPL	-	-	0.780	-	-	-	-	-
F13A01	-	-	0.720	-	-	-	-	-
D5S818	-	-	-	-	0.295	-	-	-

2005). In the present study and from the genotyping data, PE for every locus was calculated and presented in (Table 4). As expected, the power of exclusion was high for all the microsatellites analyzed it ranged from 0.372 (TPOX) to 0.820 (D18S51).

Polymorphic information content

The TPOX locus is the least polymorphic marker while D21S11 is the most polymorphic marker. The usefulness of the findings of genetic polymorphism studies and linkage mapping programs in humans is confirmed by the high PIC values of the selected markers. Similar degree of polymorphism was found in Syrian population, where the most polymorphic marker was found to be Penta E, and that the least polymorphic one was TPOX (Table 7). On the contrary, the Egyptian population showed that the FGA locus is the most polymorphic marker and that the TPOX locus, as observed in our population, is the least

Table 6. The Power of discrimination in different populations.

Locus	Iraq	Turkey	Iran	Syria	Emirates	Qatar	Egypt	Gaza
D21S11	0.969	-	-	-	-	-	-	-
D2S1338	0.960	-	-	-	-	0.973	-	-
D18S51	0.959	0.953	-	0.963	0.971	-	-	0.962
TPOX	0.759	0.772	-	-	-	0.855	-	-
Penta E	-	-	-	0.974	-	-	-	0.976
Penta D	-	-	-	0.951	-	-	-	0.961
FGA	-	-	-	0.957	-	-	0.973	0.967
vWA	-	-	-	-	-	-	0.937	-
LPL	-	-	0.924	-	-	-	-	-
F13A01	-	-	0.922	-	-	-	-	-
D5S818	-	-	-	-	0.889	-	-	-

Table 7. Polymorphic Information Content in different populations.

Locus	Iraq	Turkey	Iran	Syria	Qatar	Egypt	Gaza
D21S11	0.859	-	-	-	-	-	-
D18S51	-	0.840	-	-	-	-	-
D2S1338	-	-	-	-	0.865	-	-
TPOX	0.646	0.540	-	0.640	0.638	0.610	0.650
Penta E	-	-	-	0.890	-	-	0.900
FGA	-	-	-	-	-	0.870	-
LPL	-	-	0.770	-	-	-	-
F13A01	-	-	0.760	-	-	-	-
D5S818	-	-	-	-	-	-	-

polymorphic marker. Even in other populations like the Iran, Qatar, and Turkey, it was found that the previously mentioned loci have a similar degree of polymorphism (Ana et al., 2006; Reyhaneh and Sadeq, 2009). The polymorphic nature of microsatellites makes them the markers of choice in characterization and genetic diversity studies. The high PIC values of the selected markers confirm their usefulness for genetic polymorphism studies and linkage mapping programs in human as well. The mean heterozygosity observed, expected and mean PIC values across the 20 loci were 0.77, 0.81 and 0.78, respectively, indicating high gene diversity.

P-value: Probability value of Chi-square test for Hardy Weinberg equilibrium

Checking for HWE, it is performed by taking the observed allele frequencies and calculating the expected genotype frequencies based on the allele frequencies. If the observed genotype frequencies are close to the expected genotype frequencies calculated from the observed allele frequencies, then the population is in Hardy-Weinberg equilibrium and allele combinations are likely to be independent of one another. The results obtained from the tests for HWE is shown in (Table 4). The null hypothesis states that all the STR loci tested are in HWE and any deviation from HWE expectations is due to sampling error. The alpha value was set at 0.05. The null hypothesis was rejected if the computed p values were below the alpha value of 0.05. Therefore having a p value above 0.05 to show that the STR alleles do not differ significantly from HWE does not imply that the samples are in HWE. Butler (2005) states that “if a p-value < 0.05 is observed with a set of alleles measured at a particular STR locus, it does not mean that a laboratory should avoid using this data because it ‘failed’ a test for Hardy-Weinberg equilibrium”.

Conclusion

The results of the current study indicate these autosomal STRs Loci useful for DNA typing markers in Iraq and can be used for establishment of a DNA database that will be beneficial for the population in terms of resolving social and moral disputes and will contribute to improvements in

the justice system.

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Full Length Research Paper

Effects of different media concentartions on callogensis in sugar cane (*Saccharum officinarum L.*)

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Sugarcane (*Saccharum officinarum* L) var. 668 was propagated through tissue culture technique: callogenesis at MS media supplemented with different concentrations of growth regulators. Explants were disinfected with 70% ethanol which was sprayed directly on outer most sheathe which covered the tender stem to be used as source of explants. After 15 days, those explants which survived turned into green leaves and were used for the aforementioned study. Different concentrations of growth regulators were applied. For callogensis response from the cultivated explant, combination of Murashige and Skoog (MS) + 2,4-dichlorophenoxy acetic acid (2,4.D) + benzylaminopurine (BAP) with different concentration was applied and were found best for the establishment of callus from shoot culture.

Key words: *Saccharum officinarum* L, callogenesis, growth regulators, explants, shoot culture.

INTRODUCTION

Plant tissue culture techniques have become a powerful tool for studying and solving basic and applied problems in plant biotechnology. During the last 30 years, micro propagation and other *in vitro* techniques became more widely used in commercial horticulture and agriculture for the mass propagation of crop plants (Lal and Singh, 1994). Sugarcane (*Saccharum officinarum* L.) is an important agricultural cash crop of Pakistan and is the major source of sugar. Enhancement of sugarcane productivity mainly depends on genetic variability available and pyramiding of the potential genes (Lee, 1987). Genetic improvement in sugarcane via conventional breeding is greatly limited due to the fact that sugarcane plant scarcely flower and produce seeds under our climatic conditions (Lorenzo et al., 2001). Breeding work in sugarcane is mainly dependent on imported fuzz of inferior quality and, therefore, the available genetic base for selection of desirable genotypes is very narrow (Krisnamurthi and Tlaskal, 1974; Azeem et al., 2010). In spite of concerted efforts, a breakthrough in sugarcane yield and sucrose content could not be achieved. Likewise existing procedures for sugarcane multiplication has long been a time consuming problem in sugarcane breeding program. Micro propagation is currently the only realistic means of achieving rapid, large-scale production of disease-free quality planting material as seed canes of newly developed varieties in order to speed up the breeding and commercialization process in sugarcane. As a result of which, plant regeneration through tissue culture technique would be a viable alternative for improving the quality and productivity in sugarcane. There are reports

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Abbreviations: MS, Murashige and Skoog; **2,4-D,** 2, 4- dichlorophenoxy acetic acid; **BAP,** benzylaminopurine; **NAA,** naphthalene acetic acid.

on tissue culture of sugarcane from different countries but the first attempts to regenerate plants through *in vitro* technique were made on sugarcane by Naz (2003). Standardization of protocols for *in vitro* multiplication of sugarcane through callus culture, axillary bud and shoot tip culture have been reported by many authors (Devi and Srinivasan, 2006). However, reports are few on young meristem callus culture in sugarcane cultivar, Nayana of Orissa. The present communication demonstrates an effective high frequency regeneration method which allows for expedient multiplication of micro plants that are easily established *ex vitro* through callus culture of young meristem as an explant.

MATERIALS AND METHODS

This research project was carried out in the Nuclear Institute for Food and agriculture Turnab Peshawar (NIFA). All the facilities and equipments were provided by NIFA and Sarhad University of Science and Information Technology Peshawar (SUIT).

Maintenance of aseptic environment

Sterilization of glassware

Glassware (Petri plates, vials, culture tube, flasks, pipettes among others) and metallic instruments were sterilized in a hot air oven at 160-180°C for 2 h.

Sterilization of instruments

The metallic instruments (forceps, scalpels, needles, spatulas, among others) were first sterilized in hot air oven at 160-180°C covered by aluminum sheet and then flame sterilized, that is, dipping them in 70% ethanol followed by flaming and cooling in luminary flow.

Sterilization of nutrient media

Culture media were properly dispensed in glass container, plugged with cotton, sealed with plastic closures and sterilized by autoclaving. Minimum time required for autoclaving the nutrient media are:

Selection of explants

Explant was conducted on sugar cane *(S. officinarum L.)* collected from field grown plants.

Growth medium

MS medium (Murashiage and Skoog, 1962) supplemented with different concentrations of auxin and cytokinins along with 4% sucrose were used.

Callogenesis

Explants were obtained from shoots of sugarcane plant. The shoot was surface sterilized in 75% ethanol for 1 min, disinfected in 2% sodium hypochlorite solution for 2 min, and washed 7 times with sterile water. After removal of several outer layers of leaves, the innermost segments, starting at the base of the leaves and up to 8 cm towards the tip were cut off with a sharp sterilized scalpel. The sequential leaf segments, with a cut surface in contact with the culture medium, were placed in each Petri dish (100 x 15 mm) containing approximately 30 ml of the medium. All 48 explants were grown on the MS medium supplemented with different concentrations and combination of auxin and cytokine: MS + 2, 4-dichlorophenoxy acetic acid (2, 4-D) (2 mg/L); MS + 2, 4-D (3 mg/L); MS + 2, 4-D 2 mg/L+ benzylaminopurine (BAP) (1 mg/L) and MS + 2, 4-D 3 mg/L + BAP 1 mg/L. All cultures were incubated in the dark at 27°C ~ for callus induction and growth.

RESULTS AND DISCUSSION

Callus induction was observed within two weeks, after inoculation of the explants on MS medium (Figure 1) supplemented with 2, 4-D and BAP. Although in all concentrations of 2, 4-D the callus induction was triggered, but maximum callus growth was observed at the concentration of 3 mg/l of 2, 4-D (70%). However, more profuse callus induction was observed at 3 mg/l of 2, 4-D + 1 mg/l of BAP with full potential of callus regeneration from the explants of the cultivated varieties, Nayana. On this media composition, the explants produced creamy white callus. The percentage of callus induction was 77% (Table 1). Such type of calli has also been reported by Khan et al. (1998), Nadar et al. (1978) and Begum et al. (1995) who found that 3.5 mg/l of 2, 4-D produced highest percentage of callus induction from leaf base explant in Bangladesh Nagabari variety of sugarcane. Islam et al. (1982) and Azeem et al. (2010) also reported that 0.5-5.0 mg/l of 2,4-D showed callus induction from leaf tissue on MS medium. The concentrations of naphthalene acetic acid (NAA) at 2.0 and 3.0 mg/l produced small amount (20-30%) of callus with grayish globular and hardy in nature.

The hormone mediated callus induction and subsequent growth is dependent on certain factors, that is, appropriate concentration of sucrose and iron source among others, which may trigger the complete chain of events that influence the ability of cultured cells to grow in an organized fashion. Plant tissues, therefore, must have receptors for hormones for example GID1 and DELLA. These hormones interact with these receptors that reside either on cell membrane or within the cytoplasm. Affinity and concentration of receptors on the surface of the target tissues determine the type of response. Previously specific binding site for both auxin and cytokinin have been identified. A class of proteins called expansins mediates the proton ability to cause cell wall loosening; these expansins break the hydrogen bonds between the polysaccharide components of the wall. Proton (H+) pumping and lowering of cytosolic pH result in an elevation of intracellular calcium level. Both cytosolic pH and calcium ions act as second messengers in early auxin action. Calcium ions, either themselves and or along with calcium binding proteins, for example,

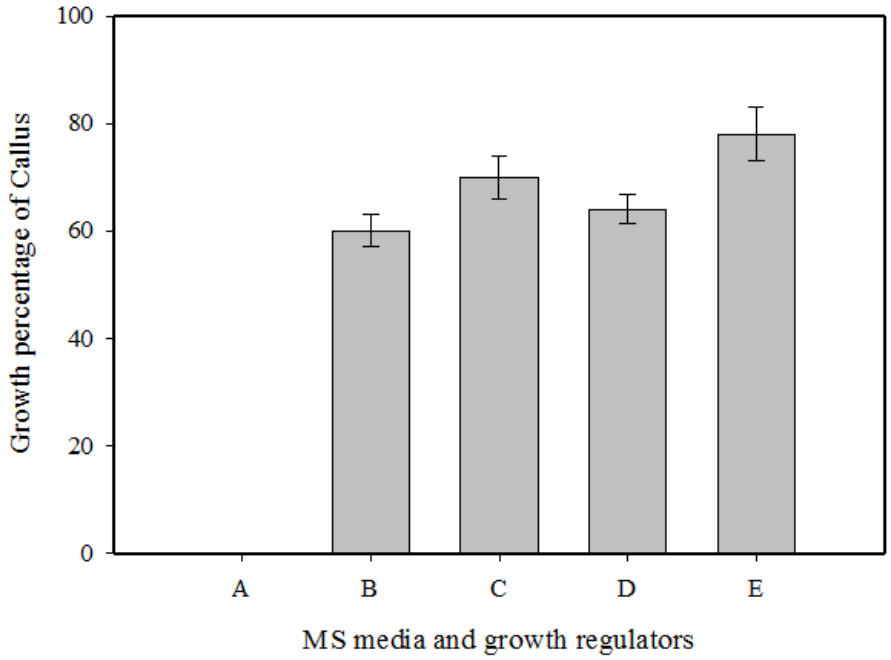

Figure 1. Healthy young meristems were collected by removing the leaf sheath from field grown plants of sugarcane (*Saccharum officinarum* L.). The explants was grown on the MS medium supplemented with different concentrations of auxin and cytokine on the callus formation in sugarcane. A, MS; B, MS + 2, 4-D (2 mg/L); C, MS + 2, 4-D (3 mg/L); D, MS + 2, 4-D (2mg/L)+ BAP (1 mg/L); E, MS + 2, 4-D (3 mg/L) + BAP (1 mg/L).

Table 1. Effect of Auxin and Cytokine's on the callus morphology, viability, totipotency and callogenesis.

Media concentration	Total number of explants culture	Callus induction	Percentage (%) Callus formation	Remark
MS + 2, 4-D (2 mg/L)	48	29	60.4	Yellowish Callus
MS + 2, 4-D (3 mg/L)	48	34	70.8	Yellowish to Brown Callus
MS + 2, 4-D (2 mg/L) + BAP (1 mg/L)	48	31	64.6	Light White Callus
MS + 2, 4-D (3 mg/L) + BAP (1 mg/L)	48	37	77.1	Light White Callus with Good Growth
MS (Basic)	48	-	-	-

calmodulin activate the protein kinase cascade which in turn activates other proteins, including the transcription factors. These factors presumably interact with the auxin-response elements and regulate the expression of auxin-inducible or auxin-responsive genes and exert its effect on cell cycle and stimulate cell division.

Conclusion

It is concluded from the present investigation that maximum growth percentages (77.1%) was obtained at 3 mg/l of 2, 4-D + 1 mg/l of BPA while exclusive 2, 4-D at the concentration of 3 mg/l maximum gave (70.1%) callus growth. So 3 mg/l of 2, 4-D + 1mg/l of BPA is suggested as ideal for maximum callus growth.

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Full Length Research Paper

Effect of overstory on the seasonal variability of understory herbs in primary broad-leaved Korean pine forest of Changbai Mountain

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In order to study the structure of herbaceous biodiversity and its effect factors in broad-leaved Korean pine forest, a total of 100 1-m^2 sub-plots were established in the 1 ha site which was monthly investigated from May to September in 2009. Data on soil attributes were also investigated (soil organic matter, available nitrogen, available phosphorus, available potassium, soil, water and pH) without light environment (leaf area index (LAI), photosynthetic photon flux density (PPFD) and, canopy presence). In this paper, the relationships between different layers (tree, shrub and herbaceous), soil attributes and light environment were analyzed. The effects of tree and shrub layers, soil attributes and light environment on the structure of herbaceous diversity were also discussed. The results show that there is an obvious seasonal dynamic for the herb in broad-leaved Korean pine forest. It was dominated by *Anemone amurensis* (Korsh.) Kom. and *Corydalis ambigua* Cham. et Schltdl. in May. The highest species richness was in June, when spring ephemeral, early and late-summer plant grew at the same time. July was the growing season and had highest diversity. From August to September, summer plant became withered gradually. There is a significant positive correlation between the diversity in shrub and herb layer Density of shrub layer positively correlated with the cover of herb layer, but negatively correlated with the diversity of herbaceous layer. The effect of the tree layer on the cover and the diversity of herbaceous layer did not arrive at the significant level. The correlation analysis of the cover, diversity and soil attributes, light environment at herbaceous layer, as well as the tree and shrub layer, indicated that available potassium, PPFD, soil water was the key connection between the tree- and shrub layer. The site locates in the broad-leaved Korean pine forest belonged to the old forest which could explain why the tree layer was not related to herbaceous layer in this paper. Therefore, it could be concluded that, the influence of the tree layer on the structure of herbaceous biodiversity carried out through the direct effect on light environment, precipitation and litter.

Key words: Tree layer, shrub layer, herbaceous layer, seasonal dynamic, soil attributes, light environment.

INTRODUCTION

Herbaceous layer in temperate forest places has an important role in energy flow and nutrient cycling in forest ecosystem. Though biomass were few represented at herbaceous layer, number of species reach the maximum

level and influences the regeneration of a canopy layer directly. Accordingly, the composition and structure of three-layer influence the herbaceous layer directly or indirectly through change in the distribution of light environment, precipitation, soil fertility as well as the physical characteristics of litter (Gilliam, 2007). The interaction between the herbaceous and tree layer leads to the linkage. This linkage existed in many forest types (Barbier et al., 2008; Antonio and Ricardo, 2009). Gilliam et al. (1995) indicates there is no connection between the herbaceous and tree layers in the infancy phase (20 years after clearing) in West Virginia, but the linkage occurs when forest mature (80~100 years). This linkage may be influenced by environmental gradient, which is confirmed by Gilliam and Roberts (2003). However, at present, the study on the effect of three-layer on the herbaceous layer is insufficient. There is no enough evidence to affirm the mechanism, until now (Stephane et al., 2008). Thus, this paper analyzed the seasonal dynamic of herbaceous diversity and their correlation with soil attributes, light environment, tree and shrub layer in broad-leaved Korean pine forest in Changbai Mountain. It aims at evaluating the structure of herbaceous biodiversity and its effect factors.

MATERIALS AND METHODS

Study site

The site is located in broad-leaved Korean pine forest of Changbai mountain Nature Reserve (N 42°20.211′, E 128°05.705′ and 784 m). The standard type is a gentle slope land belongs to the typical and the temperate continental mountain climate. It has warm and rainy summer, cold and dry winter, with annual average temperature of 3.6°C. Annual precipitation is 700 mm and concentrates between June and September. The soil type is dark brown, forest soil. Forest stand volume is 331.7 $m^3.hm^{-2}$. The density of individual (DBH ≥ 1 cm) is 1,801 $ind.hm^{-2}$. The upper of the tree-layer mainly contains *Pinus koraiensis*, *Quercus mongolica*, *Tilia amurensis*, and *Fraxinus mandshurica*. The middle of the tree layer is dominated by *Acer mono*, *Acer barbinerve*, *Corylus mandshurica*, *Syringa reticulata* which dominates the under layer of the forest. During the early spring and ephemeral spring, the main species include *Anemone amurensis*, *Corydalis spp*, and *Adonis amurensis*. During the summer and autumn, the major plant species are *Brachybotrys paridiformis*, *Cardamine leucantha* and *Meehania urticifolia*.

Data collecting

From July to September of 2005, 1 hm^2 area site was established in broad-leaved Korean pine forest used as total station. The site was divided into quadrats (20 m × 20 m) and sub-quadrate (10 × 10 m). In each quadrat, an herbaceous investigation area with 1 × 1 m as as dimension, were demarcated by spilings. Totally, 100 herb sites were set up. All the woody plants (Height ≥ 0.3 m) inside the site were numbered and tagged. The species name, height, crown and coordinates were recorded. Species, height, cover (length of the long axis and short axis); numbers of bloom and fruit in the herbaceous site were recorded during the period from May to September. Crown condition was classified by 1 (forest gap) and 0 (under the crown cover). According to the stand presented by Kudo (2008), the herbaceous strata in broad-leaved Korean pine forest can be classified into three types, early spring ephemeral, early summer plant and late summer plant. Tree layer means height of the woody plants were more than 3 m. All woody plants which height is higher than 0.3 m but less than 3 m were belong to shrub layer.

Environmental factors in this research include nitrogen, phosphorus, organic matter, PH value, soil moisture, LAI, PPFD and canopy openness. 500 g soil samples were taken from the surface layer (0 to 20 cm) in the middle of sub-quadrats interval with one. The analysis of soil samples in the lab was done according to the “Soil agricultural chemical analysis method” (Soil Science Society of China, 1999). Soil moisture was measured by HH2 DelLa-TDevices Moisture Meter (UK). PPFD was derived from the fish pictures took above 1 m in each quadrat. WinSCANOPY and XLScanopy were used to get the direct light and scattered light. LAI and canopy openness were calculated according to the method presented by Bonhomme and Chartier (1972); Zhang (2009).

Data analysis

The Shannon-Wiener diversity index and density, both in tree and shrub layer were calculated in each sub-quadrat. The monthly Shannon-Wiener diversity index, evenness and cover degree of herbaceous component were also deduced. According to Mölder’s method (2008), the raw data in May, June, July, August, and September were combined. In that regard, when given specie occurs many times in one quadrat, the biggest cover degree was chosen. The combined data stand for the all herbaceous in the growing season and used to calculate the cover degree and Shannon-Wiener diversity index. This process was carried out through R2.10.0.

Shannon-Wiener diversity index, $H = -\sum p_i \ln p_i$

Where, *H* is Shannon-Wiener diversity index, *pi* the relative frequency.

Evenness, $E = e^H / S$ (Buzas and Gibson, 1969)

Where, *E* is the evenness and *S* the species richness

In order to descript the relationship between species richness and the number of quadrats, rarefaction curve was drawn by the individual number or sample sequence (1, 2, N) as horizontal ordinate and species richness as the y-coordinate. The investigation of sample generally cannot be produced by repeated sampling (Gotelli and Colwell, 2001). The correlation among tree-, shrub- and herb layer; and interaction with the environment were implemented 999 time’s random replacement, inspection through program corPerm2 in R2.10.0 software (Legendre, 2005). The

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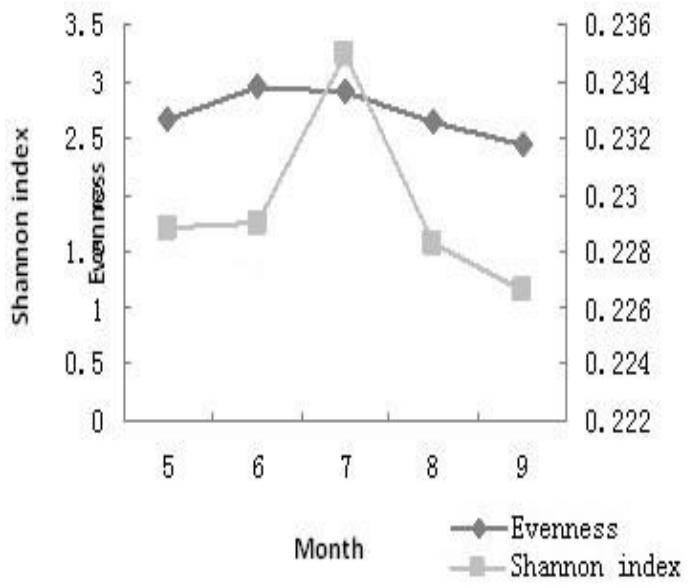


Figure 1. Seasonal dynamics of Shannon-Wiener diversity index of herbaceous plant.

Principal Coordinates of Neighborhood Matrices (PCNM) method was applied. This method is based on a principal coordinate analysis of a truncated pairwise geographic distance matrix between sampling sites. Eigen values associated with positive eigen vectors can be used as spatial predictors in multivariate regression (Dray et al., 2007). Moreover, these Eigen values have the advantage that they are orthogonal to each other and thus represent various structures over the whole range of scales encompassed by the sampling design (Borcard and Legendre, 2002). Firstly, a Euclidean distance matrix with the X and Y coordinates of each 10 × 10 m subplot was created. Then, the calculation of the principal coordinates variables was performed using the "PCNM" library (Legendre, 2007), finally, the principal coordinates variables with positive Moran's *I* value (from now on PCNM variables) was chosen.

Variation partitioning (Borcard et al., 1992) was used to discover the fraction of variation in cover and the herbaceous diversity explained by the environmental and spatial variables. The environmental matrix was created, including together the over storey related factors (tree density, canopy cover and Shannon-Wiener diversity index), shrub layers related factors (shrub density, shrub cover and Shannon-Wiener diversity index), soil attributes (soil organic matter, available nitrogen, available phosphorus, available potassium, soil water and pH) and light regime (LAI, PPFD and canopy presence). The spatial variables were created with PCNM variables. Firstly, we selected a set of environmental and spatial variables explaining a significant portion of variation in each one of the response variables (a + b and b + c, respectively). Then, we determined the fraction of variation explained by the whole set of variables (a + b + c). Partial regression was used to determine the fraction of variation explained by the environmental (fraction a) and the spatial variables (fraction c) when removing their interaction (fraction b). After that, another variation partitioning of the a + b fraction was performed to determine the relative contribution of the over storey, shrub layer, soil and light regime to this environmental fraction. The "pack for" library (Dray et al., 2007) of the R statistical language (R Development Core Team, 2007) was used to select the sets of explanatory variables. The variation partitioning analyses were carried out with the "vegan" library (Oksanen et al., 2011) and the package of the R statistical language (R Development Core Team, 2007). The environmental and response variables were transformed if needed.

RESULTS

Seasonal dynamic of diversity at herb layer

In broad-leaved Korean pine forest, the higher plant included 13 early spring ephemeral species, 37 early summer plants and 21 late summer plants. The type of species varied with the season. In May snow just melting, there are lots of early spring ephemeral species like *A. amurensis*, *Corydalis spp*, *A. amurensis*, early summer plants, *Hylomecon japonica*, *Paris quadrifolia*, *Veratrum nigrum*, and few late summer plants, *Lilium distichum*, *Filipendula palmate*, *Cacalia hastate*. In June, there was still a little early spring ephemeral species, lots of early summer plants and some late summer plants growing. Therefore, the highest species richness happened in this month. From July to September, the early summer plants and late summer plants withered gradually with the decreasing of the species number. Different from the number of species, the Shannon-Wiener diversity index in July was the highest one (Figure 1). From May to September, the evenness index change un-conspicuously. Rarefaction curve showed that with the increasing of number of quadrat, species richness increased gradually. When the number of quadrat was less than 30, the species richness increased sharply. Rarefaction curve and species number were in accordance. The highest species richness occurred in June, and lowest one was in September. There were 26 woody plants (height ≥ 3 m) and 32 woody plants (0.3 m ≤ height < 3 m) in the tree and shrub layer, respectively. There were 21 common species between the tree- and shrub-layer, including small trees, sapling and part of tall shrub. The correlation analysis between the density, cover degree and diversity of tree and shrub layer; and the cover degree and diversity at herb layer show that there was a significant positive correlation between the diversity and the density (Table 1). It was for tree- and shrub layer. The diversity between the herbaceous layer and shrub layer reach the significant positive correlation at the level of 0.01.

The analysis among the environmental variables (Table 2) indicates that there were significant positive correlations among content of organic matter, available phosphorus, and available potassium at the level of 0.001. Soil moisture negatively related with organic matter. Soil acidity and alkalinity is positively related with available phosphorus and available potassium. LAI index significantly is positively correlated with PPFD at the level of 0.01, but significantly, negatively correlated with soil

Table 1. Correlation coefficients between density (D), coverage (C) and diversity (H) of the tree and shrub layers, and coverage (C) and diversity (H) of herbaceous layers.

Parameter	Tree D	Tree C	Tree H	Shrub D	Shrub C	Shrub H	herbaceous H	Herb C
Tree D	1	-0.068	0.322**	0.094	0.105	0.159	0.069	0.074
Tree C		1	0.089	0.068	0.031	0.112	0.128	-0.11
Tree H			1	-0.102	0.156	0.141	0.046	-0.074
Shrub D				1	0.081	0.248*	-0.01	0.098
Shrub C					1	0.013	-0.102	-0.14
Shrub H						1	0.242**	-0.009
herbaceous H							1	0.127
herbaceous C								1

*P < 0.05, **P < 0.01, ***P < 0.001.

Table 2. Correlation coefficients between the nine quantitative environmental variables.

Parameter	O.M	N	P	K	Soil water	pH	PPFD	LAI	Gap
O.M	1	-0.183	0.521***	0.357***	-0.308**	0.016	0.120	0.060	-0.054
N		1	0.140	0.231*	0.007	-0.175	0.086	-0.077	-0.071
P			1	0.668***	0.017	0.341**	0.182	0.158	0.098
K				1	0.020	0.421***	0.143	-0.091	0.025
Soil water					1	0.076	-0.214*	-0.231*	0.390***
pH						1	0.023	-0.230*	0.073
PPFD							1	0.286**	-0.006
LAI								1	-0.022
Gap									1

*P < 0.05, **P < 0.01, ***P < 0.001.

Table 3. Relationship between species richness (SR), Shannon-Wiener diversity index (H), and coverage (C) of three layers (tree, shrub, and herbaceous layer) and environmental parameters, respectively.

Parameter	O.M	N	P	K	Soil water	pH	PPFD	LAI	Gap
Tree number	-0.270**	0.017	-0.282**	-0.290**	0.009	-0.184	-0.108	0.005	0.161
Tree C	0.092	-0.236*	-0.215*	-0.148	-0.205*	-0.094	0.082	-0.058	-0.083
Tree eH	-0.181	0.096	-0.277*	-0.120	-0.113	-0.091	-0.130	-0.063	-0.140
Shrub number	-0.074	-0.188	-0.112	-0.137	0.107	-0.034	-0.158	-0.088	0.279**
Shrub C	-0.162	0.064	-0.163	0.032	-0.049	-0.033	0.068	0.220*	-0.001
Shrub H	-0.110	-0.094	-0.216*	-0.269**	-0.031	-0.214*	-0.198*	0.004	-0.152
herbaceous H	-0.161	-0.050	-0.131	-0.081	0.149	-0.069	-0.261*	-0.183	-0.270**
herbaceous C	-0.035	0.095	0.279**	0.398***	0.436***	0.227*	-0.065	-0.311**	0.294**

* P < 0.05, **P < 0.01, ***P < 0.001.

moisture and PH value. Soil moisture significantly is positively correlated with forest gaps at the level of 0.001.

The analysis between three layers (tree, shrub and herbaceous layer) and environmental factors (Table 3) showed that soil organic matter and tree density were significantly and negatively correlated. Available nitrogen significantly related to tree cover degree. Available phosphorus is significantly and negatively correlated with the density, cover degree and diversity in tree layer and diversity at shrub layer, while positively related to the cover degree at herbaceous layer. Available potassium is significantly and negatively correlated with the density,

cover degree and diversity at tree-layer and diversity at shrub layer, but positively related with the cover degree at herbaceous layer. The cover degree was significantly and positively related with soil moisture at the level of 0.001, but negatively with LAI index. In addition, the cover degree at herbaceous layer and density at shrub layer were both positively related with forest gaps at the level of 0.01. Above all, soil nitrogen and potassium were significantly correlated on the environmental factors coexisting at tree, shrub and herbaceous layer. The density of shrub layer and cover degree of herb-layer was positively related to forest gaps; that is, the distribution of tree crown. At three different layers, the effect of environmental factors on the density of tree layer, the diversity of shrub layer and cover of herbaceous layer was the most obvious.

Result of variation partitioning taking monthly herbaceous coverage and diversity as the dependent variable respectively, and environment variables and space variables (explanatory variables) showed that available potassium and soil moisture which were screened from the environmental factors could explain 12.0% of variation. Spatial variable simply could explain 1.9% of the variation. The environmental factors and spatial variable could totally explain 19.4% of variation (Figure 2a). Available potassium and forest gaps screened from the environmental factors explained 7.5% of diversity variation at herbaceous layer during the whole growing season. The environmental factors and spatial variable could totally explain 28.5% of variation (Figure 2b). The analysis of monthly cover degree and diversity showed that in May the significant influencing factors on cover degree was the density of shrub. However, variable potassium also had a certain effect without reaching the significant level. The environmental factors could simply explain 3.0% of variation. The environmental factors and spatial variable could totally explain 29.6% of variation (Figure 2c).

The extremely significant factors influencing on diversity at herbaceous layer were available in potassium, LAI and forest gaps. The environmental factor composed by them could explain 16.5% of diversity variation. The environmental factors and spatial variable could totally explain 39.8% of variation (Figure 2d). In June, the extremely significant factors influencing on diversity at herbaceous layer where available potassium and shrub density, which could explain 8.3% of the variation. The environmental factors and spatial variable could totally explain 17.9% of variation (Figure 2e). The main effects of herb diversity where available potassium and shrub density, which could explain 7.5% of the variation. The environmental factors and spatial variable could totally explain 19.1% of variation (Figure 2f). In July, shrub density, available potassium and LAI had an extreme and significant impact on herb cover degree. Tree density has also significantly effect on herbaceous cover degree.

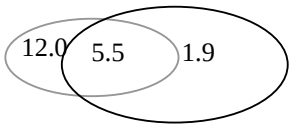
Environmental factors could explain 22.9% of the variation. The combination of environmental and spatial variable could explain 32.1% variation (Figure 2g). The herbaceous diversity significantly related to forest gaps, available potassium and PPFD in this month. The composition of them could explain 13.3% of diversity variation. The environmental and spatial factors totally explain 24.2% of variation (Figure 2h). Variation partitioning was not implemented in August, because there was no significant correlation between the cover degree and diversity at herbaceous layer and each environmental factor. In September, LAI and forest gaps had a significant impact on the herb cover degree. The influence of PPFD decreased. The environmental variation composited by them could explain 14.0% of the variation. The environmental and spatial factors totally explain 20.8% of variation (Figure 2i). In contrast, the shrub density and diversity had an impact on the herb diversity and did not reach to the significant level. Thus, the density and diversity at shrub-layer could only explain 5.1% of diversity variation. The environmental and spatial factors totally explained 14.8% of variation (Figure 2j). All the above results suggested that the environmental factors influencing the herbaceous cover degree were different accordingly to the period. Extremely and significantly potassium available is correlated with the herb cover degree, but negatively correlated with herbaceous diversity in the early and middle of the growing season.

DISCUSSION

The results show that the diversity at shrub layer was extremely and significantly positive with the herbaceous layer. The density at shrub layer was positive with the cover degree at herbaceous layer, but negative with the herb diversity. The effect of the tree layer on cover degree and diversity at herbaceous layer did not arrive at the significant level. As the species below in the canopy, the negative relation between the number of shrub and diversity at herbaceous layer were accepted (Kwiatkowska, 1994; Godefroid et al., 2005; Baker and Van Lear, 1998; Hicks, 1980). In accordance with the previous results (Ewald, 2000; Neumanna and Starlinger 2001; Aubert et al., 2004), the cover degree was not significantly related to the diversity at herbaceous layer. In addition, the fact that diversity at tree layer was positively related with the one at an herbaceous layer and this was also found (Hicks, 1980; McCune and Antos, 1981; Bradfield and Scagel, 1984; Ingerpuu et al., 2003; Mölder et al., 2008). However, previous research showed that the effect of the tree layer on the herbaceous layer was significant and positive in the “general stand” (exclude of old forest and planted forest), but did not reach the significant level in the old forest and planted

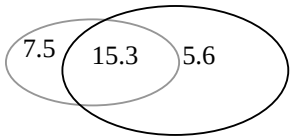
a) The all herbaceous coverage

Environmental factors:
[a+b]=17.5***
Spatial factors:
[b+c]=7.4**
Residual=80.6
K:（0.398***）
Soil water（0.286**）
PCNM：6,14



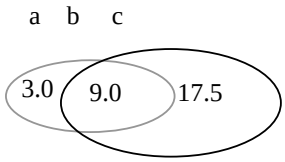
b) The all herbaceous H

Environmental factors:
[a+b]=22.9***
Spatial factors:
[b+c]=20.9***
Residual=71.5
K:（-0.346**）
Gap（-0.265*）
PCNM：4,6,13,14,15,18



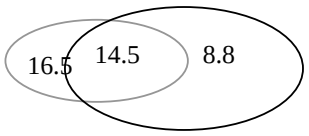
c) Herbaceous coverage of May

Environmental factors:
[a+b]=12.0***
Spatial factors:
[b+c]=26.5***
Residual=70.4
Shrub density（0.359**）
PCNM：14, 16, 18, 20



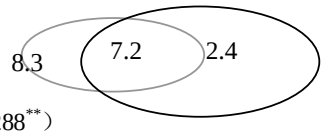
d) Herbaceous H of May

Environmental factors:
[a+b]=31.0***
Spatial factors
[b+c]=23.3***
Residual=60.2
LAI（0.324**）
Gap（-0.292**）
K:（-0.415***）
PCNM：4, 6, 14, 19



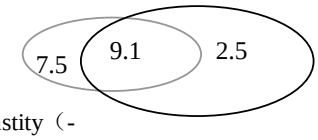
e) Herbaceous coverage of June

Environmental factors:
[a+b]=15.5***
Spatial factors:
[b+c]=9.6***
Residual:=82.1
K:（0.302**）
Soil water（0.288**）
PCNM：6, 13



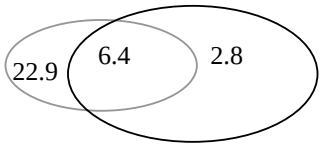
f) Herbaceous H of June

Environmental factors
[a+b]=16.6***
Spatial factors
[b+c]=11.6**
Residual=80.9
K（-0.289**）
Shrub denstity（-0.223*）
PCNM：4, 13, 14



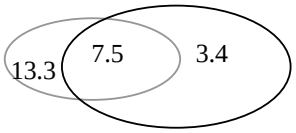
g) Herbaceous coverage of July

Environmental factors:
[a+b]=29.3***
Spatial factors:
[b+c]=9.2***
残差=67.9
LAI（-0.321***）
K（0.278**）
Shrub density（0.342**）
Tree density（0.239*）
PCNM：6



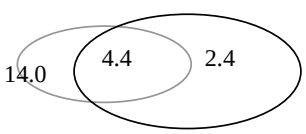
h) Herbaceous H of July

Environmental factors:
[a+b]=20.8***
Spatial factors:
[b+c]=10.9**
Residual=75.8
Gap（-0.353**）
K（-0.295**）
PPFD（-0.197*）
PCNM：6, 23



i) herbaceous coverage of September

Environmental factors:
[a+b]=18.4***
Spatial factors:
[b+c]=6.8**
Residual=79.2
LAI（-0.247*）
Gap（0.310**）
PPFD（0.153）
PCNM：13, 24



j) herbaceous H of September

Environmental factors
[a+b]=5.1*
Spatial factors:
[b+c]=9.7***
Residual=85.2
Shrub density（-0.122）
Shrub eH（0.197）
PCNM：13, 16

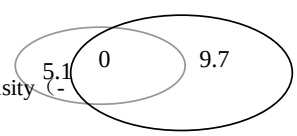


Figure 2. Result of variation partitioning (H: Shannon-Wiener diversity index).

forest. The site locates in the broad-leaved Korean pine forest belonged to the old forest which could explain why the tree layer was not related to herbaceous layer in this paper.

Results of variation partitioning indicated that available potassium were extremely and significantly positive with the herbaceous cover degree in the early and middle of the growing season but negatively with the diversity. Self-correlation analysis of soil and light available showed that available potassium, soil organic matters, available phosphorus and pH were extremely and significantly positive at the level of 0.001, but they extremely and significantly negative with tree density at the level of 0.01. Available potassium was extremely and significantly negative with shrub density. This suggested that available potassium was the key factor to connect the three layers. The possible reasons were the tree layer influenced the distribution of organic matters, potassium and phosphorus in the soil, which in turn effect the distribution of herb. The advantage herbaceous grew well in the area rich in nutrition. Numbers and cover degree of individual increased, leading the greatest cover degree at herbaceous layer. Oppositely, the advantage's growth was restricted in the area lacking nutrition. It provided chances for other species' invading and made herb diversity to be higher.

Light condition was considered as the limited factor for the coverage and richness of vegetation (Hill, 1979; Kirby, 1988; Bazzaz, 1990; Jennings et al., 1999). The light environment under the canopy was determined by canopy structure, which also controlled the temperature and moisture under the forest, especially for the density of the forest crown (Sharpe et al., 1996). Environmental factor under different species was different (Porte et al., 2004) which influenced the vegetation under the forest. It may be due to the temperature and moisture related to the light condition (Nihlgard, 1969). The light under the storey could be a comprehensive factor which stands for the difference of this microenvironment. Most research explained the effect of tree-layer on herbaceous layer through the light environment. In this paper, PPFD, LAI and forest gap were used as the light indicator. Results showed that the herbaceous cover degree and existence of forest gap were positive with the PPFD, but negative with the LAI in September. However, the diversity of herb was negative with the existing forest gap in May and July. Under the forest gap, there was enough light to improve the growth of advantage herbaceous and enlarge the herbaceous cover. On the other hand, Shannon-Wiener diversity index decrease because of the difficult invading of disadvantage species. Therefore, the relationship between the herb coverage and forest gaps was the opposite of the herb diversity. It could be concluded that light environment was also an important factor which influences the herbaceous coverage and diversity.

Soil moisture has an extremity and positive response

both to herbaceous coverage and forest gaps, but negatively related to the tree coverage and content of organic matters. It suggested that soil moisture is another important factor connecting with tree and herbaceous layer. Soil moisture was relevant with coverage at tree layer and forest gaps, which indicated that the distribution of forest crown had an impact on the distribution of light environment and precipitation, thereby on the growth of herbaceous plants.

Hill (1979) considered that the mechanism of tree layer impact on herbaceous layer was through light, precipitation and litter content. In this paper, it was found that organic matters, nitrogen, phosphorus, potassium, soil, water and light factor were strongly relevant with tree and herb layer. It indicated that though the density, coverage, and diversity at the tree layer did not have a direct impact on the coverage and diversity at herbaceous layer, it effects the distribution at herbaceous layer through changing the distribution of soil nutrient, water and light.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Air pollution tolerance indices of some plants around Ama industrial complex in Enugu State, Nigeria

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Green house gases pollute the atmosphere and cause global warming which has become a worldwide environmental problem. In order to evaluate the ability of plants to absorb such gases, the air pollution tolerance indices (APTI) of some plants around an industrial complex in Nigeria, were determined. The total chlorophyll, ascorbic acid, pH, and relative water content of the leaf extracts of *Mangifera indica*, *Delonix regia*, *Bougainvillea spectabilis*, *Ixora coccinea*, *Anacardium occidentale* and *Duranta erecta* were used to determine the APTI. *Delonix regia* gave significantly ($p<0.05$) highest APTI (5.308 ± 0.090), followed by *Bougainvillea spectabilis* (4.904 ± 0.001) and *Duranta erecta* (4.577 ± 0.166). *Anacardium occidentale* had the lowest (3.470 ± 0.001). *Delonix regia* was comparatively the most tolerant to air pollution. It is suggested that plants with high APTI values should be grown near pollution - prone areas to absorb and thus, screen off certain harmful gaseous pollutants which contribute to green house effect, global warming and climate change.

Key words: Air pollution tolerance index, ascorbic acid, chlorophyll, pH, water, plants.

INTRODUCTION

Emission of green house gases is one of the major problems arising from human population explosion and industrialization. The use of fossil fuels such as petroleum hydrocarbons and coal for transport, electricity generation for industries and households; land clearing, deforestation, agriculture and land use, produce large quantities of oxides of carbon, nitrogen and sulphur, as well as methane, aerosol particulates, etc. These pollute the environment; destroy the atmospheric ozone shield that protects organisms from high levels of ultraviolet radiation, resulting in global warming and climate change (IPCC, 2007).

Many workers like Chauhan (2010), Singh et al. (1991), Abida and Harikrishna (2010) and Sirajuddin and Ravichandran (2010) used ascorbic acid, chlorophyll, relative water content and leaf extract pH to evaluate the susceptibility of some plants to air pollutants by computing these four physiological parameters together in a formation signifying their air pollution tolerance index (APTI). Plants with higher APTI values are more tolerant to air pollution than those with low APTI values. Those with low APTI values are sensitive plants and may act as bio-indicators of pollution (Chandawat et al., 2011, Shannigrahi et al., 2004). Hence, on the basis of their indices, different plants may be categorized into tolerant, moderately tolerant, intermediate and sensitive plants (Chandawat et al., 2011). Ascorbic acid is an antioxidant, which contributes in protecting the plants against oxidative

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damage resulting from aerobic metabolism, photo-synthesis and a range of pollutants (Lima et al., 2000). Reactive oxygen species are produced in plants after exposure to environmental conditions like drought, cold or air pollution. Plants sense drought conditions and air pollution by building up reactive oxygen species and then respond by reducing the amount of water that escapes from their leaves (Gallie and Chen, 2004). The water content of the plant tissues helps to maintain the physiological balance of the plant when subjected to the stress of air pollution. Hence, the water content is related to the degree of pollution. The pH of the plant tissue is also related to the degree of air pollution since air pollutants interact with rainwater to form mixtures and solutions with varying pH, depending on the type of pollutant. Chlorophyll is involved in the productivity of the plants and its level is a direct measure of leaf damage by pollution (Bayon et al., 1989; Heath, 1989). Its measurement is an important tool for evaluating the effects of air pollutants on plants since it plays an essential role in plant metabolism and any reduction in chlorophyll content corresponds directly to plant growth (Joshi and Swami, 2009).

The objective of this work is to determine the air pollution tolerance index (APTI) of some plants so as to identify those that may be planted around polluted areas in order to attenuate the adverse effects of pollutants on man and other organisms. The screening of plants globally or in Nigeria for their APTI values has not been exhausted. So there is the need to carry out more work on numerous plants so as to help mitigate the effects of air pollution globally since air pollution has no political boundaries.

The experimental plant species used for this work were collected from Ama industrial complex, 9th mile corner, Ngwo, Udi Local Government Area (L.G.A.), Enugu State, Nigeria. 9th mile corner is a small fast growing city that is located about nine miles before Enugu, the capital of Enugu State, Nigeria. Industries such as bottling companies, breweries, table water purifying industries, hospitals, vehicle mechanic sites, as well as small and medium scale industries are located in it. It is often used as a stop-over town for heavy duty vehicles, tankers, trailers, etc. that ply from the Northern part of Nigeria to the South. Consequently, gaseous emissions and particulate matter are often released by the industrial plants, vehicles and refuse dumps and these pollute the atmosphere around the area.

MATERIALS AND METHODS

Plant collection

Leaves of *Mangifera indica* L. (Family Anacardiaceae), *Delonix regia* (Hook.) Raf. (Family Leguminosae - Caesalpiniodeae), *Bougainvillea spectabilis* Comm. ex Juss. (Family Nyctaginaceae), *Ixora coccinea* L. (Family Rubiaceae), *Anacardium occidentale* L. (Family Anacardiaceae) and *Duranta erecta* L. (Family Verbenaceae) were collected from around some industries in Ama, 9th mile corner, Ngwo, Udi L.G.A., Enugu State, Nigeria (06°.26N and 007°.23E), in the morning between 9 am and 11 am local time. The plants selected were those available at the experimental site and they have similar light, water and soil conditions. Control plants were collected from a non-industrialized area, namely, Botanical Garden, University of Nigeria, Nsukka. The plants were identified by a taxonomist, Mr. Alfred Ozioko. Samples were transported to the laboratory in a water proof container and the fresh weights of the leaves were taken immediately. Some were dried, pulverized and preserved at 4°C for further analysis.

pH determination

pH determination was carried out according to the method of Shannigrahi et al. (2004).

Ascorbic acid determination

Spectrophotometric method was used to determine the ascorbic acid content of the leaves (Abida and Harikrishna, 2010). 1 g of ground fresh leaves was homogenized in 4 ml oxalic acid - ethylenediaminetetraacetic acid (EDTA) extracting solution, for 30 s. 1 ml of orthophosphoric acid and 1 ml 5% tetraoxosulphate (vi) acid were added. 2 ml of ammonium molybdate and 3 ml of water were also added. The solution was left to stand for 15 min. The absorbance was read off with a CE 234 31D digital spectrophotometer at 760 nm. The concentration of the ascorbic acid was determined from a standard ascorbic acid regression curve.

Total chlorophyll determination

For the total chlorophyll determination, 3 g of fresh, ground leaves were extracted with 10 ml of 80% acetone and left to stand for 15 min. It was filtered and centrifuged at 2,500 rpm for 3 min. The absorbance of the supernatant was read at 645 nm (D_{645}) and 663 nm (D_{663}) using a CE 234 31D digital spectrophotometer. The optical density of the total chlorophyll (OT) is the sum of chlorophyll a (D_{645}) density and chlorophyll b (D_{663}) density, thus:

$OT = 20.2 (D_{645}) + 8.02 (D_{663})$

Total chlorophyll (mg/g DW) = 0.1 OT × (leaf DW ÷ leaf fresh weight) (Liu and Ding, 2008).

Relative leaf water content

The relative leaf water content was obtained by the method of Liu and Ding (2008) and Gharge and Menon (2012).

Determination of air pollution tolerance index (APTI)

Using these four parameters, the air pollution tolerance index (APTI) for each of the plant species was determined using the following mathematical formula:

$$APTI = \frac{A(T + P) + R}{10}$$

Where, A = Ascorbic acid (mg/g), T = Total chlorophyll (mg/g), P = pH of the leaf extract, R = relative water content (%) (Rai et al., 2013).

Table 1. Air pollution tolerance index (APTI) of some plant species around some industries in Ama, Enugu State, Nigeria.

Plant specie	Site	Total chlorophyll (mg/g)	Ascorbic acid (mg/g)	pH	Relative water content (%)	APTI
Mangifera indica	Exp.	1.495 ± 0.000^{f}	0.042 ± 0.002^{b}	6.3 ± 0.000	$4.181 \pm 0.066^{a,b}$	4.508 ± 0.002^{b}
	Cont	0.058 ± 0.002^{a}	0.012 ± 0.001^{a}	5.1 ± 0.000	3.261 ± 0.007^{d}	3.323 ± 0.002^{f}
Delonix regia	Exp.	0.901 ± 0.000^{e}	0.044 ± 0.001^{b}	5.7 ± 0.000	5.190 ± 0.002^{c}	5.308 ± 0.090^{e}
	Cont	0.093 ± 0.002^{b}	0.015 ± 0.002^{a}	7.1 ± 0.000	3.765 ± 0.002^{e}	3.873 ± 0.002^{g}
Bougainvillea spectabilis	Exp.	2.274 ± 0.002^{i}	0.028 ± 0.002^{e}	4.3 ± 0.000	$4.219 \pm 6.67^{a,b}$	4.904 ± 0.001^{d}
	Cont	0.900 ± 0.001^{e}	0.012 ± 0.001^{a}	7.6 ± 0.000	3.121 ± 0.004^{f}	3.223 ± 0.001^{h}
Ixora coccinea	Exp.	0.216 ± 0.001^{h}	0.044 ± 0.003^{b}	4.8 ± 0.000	3.507 ± 0.037^{a}	3.728 ± 0.004^{a}
	Cont	0.080 ± 0.013^{b}	0.034 ± 0.001^{d}	5.1 ± 0.000	3.411 ± 0.003^{g}	3.587 ± 0.002^{j}
Anacardium occidentale	Exp.	1.066 ± 0.000^{g}	0.051 ± 0.002^{c}	4.6 ± 0.000	3.441 ± 0.004^{g}	3.470 ± 0.001^{i}
	Cont	0.379 ± 0.003^{d}	0.038 ± 0.001^{d}	4.5 ± 0.000	3.170 ± 0.003^{f}	3.355 ± 0.000^{f}
Duranta erecta	Exp.	0.347 ± 0.000^{j}	0.053 ± 0.003^{c}	5.3 ± 0.000	$4.547 \pm 1.667^{b,c}$	4.577 ± 0.166^{c}
	Cont	0.199 ± 0.001^{c}	0.042 ± 0.001^{b}	5.8 ± 0.000	3.831 ± 0.002^{h}	4.083 ± 0.000^{k}

Exp. = Experimental plants, Cont = Control plants. Values represent means ± standard error. Means followed by the same letters in the same column are not significant at $p<0.05$.

The tolerant plants were calculated as those with APTI more than the mean APTI + SD (standard deviation). The sensitive plants were those with APTI less than mean APTI - SD, while the moderately tolerant plants have intermediate values between that of the tolerant and sensitive plant species (Liu and Ding, 2008). Three replicate samples were taken.

Statistical analysis

The data were subjected to analysis of variance (ANOVA). Multiple comparisons were made between treatment means using Duncan's multiple range tests at $p<0.05$ confidence level (Edafiogho, 2006). t - Tests were carried out between the experimental data and their controls.

RESULTS

The results of the air pollution tolerance index (APTI) determined for the six plants showed that *Delonix regia* gave significantly ($p<0.05$) highest APTI value of 5.308 ± 0.090, followed by *Bougainvillea spectabilis* (4.904 ± 0.001) (Table 1). The plant with significantly ($p<0.05$) lowest APTI value was *Anacardium occidentale* (3.470 ± 0.001). The plant species from the polluted area in Ama Industrial Complex gave consistently and significantly ($p<0.05$) higher APTI values, total chlorophyll, ascorbic acid and relative water content than plants from relatively unpolluted (control) site. For pH, *D. regia, B. spectabilis, I. coccinea* and *D*. erecta from the polluted site were comparatively more acidic than their counterparts from the control site. However*, M. indica* and *A. occidentale* from the polluted site were less acidic than those from the control site. *D. regia* and *B. spectabilis* from the control site were slightly alkaline. Among the experimental plants, *B. spectabilis* had the highest chlorophyll content, followed by *M. indica*, while *I. coccinea* had the lowest chlorophyll content ($p<0.05$ significance). Among the test plants, the leaf extract of *D. erecta* gave the highest quantity of ascorbic acid, followed by that of *A. occidentale*, while *B. spectabilis* gave the lowest ($p<0.05$ significance). Among the experimental plants, *D. regia* had the highest percentage (%) relative water content, while *A. occidentale* had the lowest ($p<0.05$ significance).

DISCUSSION

The results demonstrate that different plant species respond in different ways to air pollution and the same plant species growing in different environments may respond differently, depending on the level of air pollution in the habitat. Hence, plants possess different pollution tolerance capabilities depending on the species and the environmental factors affecting them. In the present work, the variation in the APTI values could be attributed to the different responses of the plants to the four physiological factors, namely, ascorbic acid, total chlorophyll, pH of the leaf extract and the relative water content of the leaf. These physiological factors, in turn, are affected by variation in the level of air pollution in the environment. *D. regia* was the most tolerant plant. Moderately tolerant plants were *B. spectabilis, D. erecta* and *M. indica*. Sensitive ones were *I. coccinea* and *A. occidentale*, in the present investigation. Rai et al. (2013) reported that

Mangifera indica and *Bougainvillea spectabilis* were tolerant for both industrial and non industrial sites in India. Gharge and Menon (2012) showed a decreasing order of tolerance to air pollution as: *Alternanthera sessilis* > *Amaranthus spinosus* > *Chenopodium album* and *Eclipta alba*. Chandawat et al. (2011) also reported that *Ficus benghalensis* gave the highest APTI in Ahmedabad city, India, followed by *Ficus religiosa* ≥ *Ficus glomerata*, followed by *Azadiracta indica* ≥ *Polyalthia longifolia*. They emphasized that plants with high APTI can serve as tolerant plants, while those with low APTI can serve as sensitive ones. Agbaire and Esiefarienrhe (2009) reported that *Emilia sonchifolia, Manihot esculenta* and *Elaeis guineensis* were the more tolerant species around Otorogun gas plant in Delta State, Nigeria. Liu and Ding (2008) found a number of tolerant to moderately tolerant plants near a Beijing steel factory, China and these included *Cotinus coggygria, Periploca sepium, Lespedeza floribunda* and *Grewia biloba*. The comparatively high APTI values recorded for *Delonix regia, Bougainvillea spectabilis, Duranta erecta* and *Mangifera indica* in the present work, is in agreement with the report of Shannigrahi et al. (2004) who found high APTI values for *Mangifera indica, Moringa pterydosperma, Cassia renigera* and *Ailanthus excelsa*. Plants with higher APTI (tolerant plants) can trap and contain dust particles or smog, absorb pollutants, heat, other gaseous emissions and improve the ambient air quality. Such plant species should be grown in polluted cities, along roads and around industrial areas to create a sort of “curtain” that will absorb pollutants and screen the environment from their harmful effects. The plant species from Ama Industrial complex showed significantly ($p<0.05$) higher values of APTI than the plant species from comparatively less polluted area (control), in the present investigation. Nwadinigwe (2009), Gharge and Menon (2012) and Rai et al. (2013) reported an increase of APTI values of plants at the experimental site when compared with those at the control site. This may be due to constant exposure of these plants to emissions of gaseous and particulate matter from industries operating where they were collected, as well as emissions from vehicle exhausts.

The plant species from the polluted site gave higher values for ascorbic acid, total chlorophyll and relative water content than the control plants. Perhaps the plants exposed to air pollution are naturally adjusting to these gaseous pollutants by increasing these physiological parameters in an attempt to contend with the environmental pollution. Dohmen et al. (1990) observed that when exposed to air- borne pollutants, most plants experience physiological changes before exhibiting visible damage to the leaves. The ascorbic acid from plants in the polluted site was higher than that from the control plants, in this present work. This agrees with the reports of Chandawat et al. (2011), Meerabai et al. (2012) and Rai et al. (2013) who found higher levels of ascorbic acid in the leaves of the most tolerant plants and those at the polluted sites and this suggests their tolerance to the air pollutants. The lower ascorbic acid in the leaves of the sensitive ones supports the sensitive nature of the plants to the pollutants. Ascorbic acid is important in cell wall synthesis, defence and cell division (Conklin, 2001). It is a strong reductant and it activates many physiological and defence mechanisms. It plays an important role in photosynthetic carbon fixation (Pasqualini et al., 2001). Due to its importance, it is used as a multiplication factor in the formula used in deriving APTI (Liu and Ding, 2008).

In the present work, the total chlorophyll of the test plants was higher than those of the control plants. Agbaire and Esiefarienrhe (2009) reported similar results in their work around Otorogun gas plant. Jyothi and Jaya (2010) observed higher levels of total chlorophyll in *Ficus benghalensis* and this may be due to the tolerant nature of the plant. On the other hand, Gharge and Menon (2012) and Rai et al. (2013) reported lower chlorophyll content of plants from experimental site when compared with those from the control. Certain pollutants increase the total chlorophyll content while others decrease it (Allen et al., 1987). Chandawat et al. (2011) observed that the chlorophyll content of all plants they tested varied with the pollution status of the area, as well as the tolerance and sensitivity of the plant species. The total chlorophyll is related to ascorbic acid productivity since the ascorbic acid is concentrated mainly in the chloroplasts (Liu and Ding, 2008).

In the present investigation, the relative water content of the leaves from the polluted area was higher than those from the control plants. It is similar to the report of Agbaire and Esiefarienhe (2009), Gharge and Menon (2012) and Rai et al. (2013), who found higher relative water content in the experimental plants than in the control plants. A high water content in a plant’s body helps to maintain its physiological balance in stress conditions such as exposure to air pollution (Verma, 2003), when the transpiration rates are usually high. High relative water content contributes to the normal functioning of biological processes (Meerabai et al., 2012) and favors drought and pollution resistance in plants.

The result of pH of the leaf extract was variable, in the present investigation. High pH may increase the efficiency of conversion from hexose sugar to ascorbic acid (Escobedo et al., 2008), while low leaf extract pH showed good correlation with sensitivity to air pollution (Yan-Ju and Hui, 2008). This agrees with the present investigation, where *I. coccinea* and *A. ocidentale* that were found to be sensitive plants, had comparatively low leaf extract pH. Similarly, *D. regia, B. spectabilis, I. coccinea* and *D*. erecta from the polluted site were comparatively more acidic than their counterparts from the control site, in the present work. Rai et al. (2013) found that plants from the industrial site had a pH towards the acidic side, whereas those from the non industrial site showed neutral to slightly alkaline range.

Combining a number of physiological parameters in the determination of APTI gives a more reliable result than depending on a single biochemical or physiological factor (Liu and Ding, 2008). Air pollution tolerance is affected by natural climatic conditions such as rainfall, temperature, soil type, relative humidity, etc. and these were not taken into consideration in this work, since all the experimental plants were collected from the same climatic environment. The same applies to the control. The variation in the APTI values could be attributed to the different responses of the plants to the physiological factors and these receive most impact from the pollution load in the environment. APTI is an inherent quality of plants to encounter air pollution stress (Rai et al., 2013). These physiological factors help plants to adjust to stresses in the environment, especially pollution, drought, fire, etc. and the major stress observed in the environment was that of pollution.

Conclusion

Tolerance of plants to air pollution may be specific to the site depending on the level of pollution. Plants growing in the industrialized area have higher APTI than those at the non industrial site. Plant species (such as *Delonix regia, Bougainvillea spectabilis, Duranta erecta* and *Mangifera indica*) that are tolerant to air pollution can absorb air pollutants, particulate matter and other emissions, thereby improving the air quality which man and other organisms are exposed to. Such plants should be grown on sites exposed to air pollutants, industrial areas, cities and along the roads. Sensitive plants (such as *I. coccinea* and *A. occidentale*) are more useful as bioindicators (Agrawal et al., 1991). Therefore, more work should be carried out on the APTI determination of many more plants globally, since air pollution is a global menace.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Effect of road side dust pollution on the growth and total chlorophyll contents in *Vitis vinifera* L. (grape)

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The effect of dust in *Vitis vinifera* L. on its pigmentation and growth was studied in 2012. Measurements were taken for plants in the Campus University of Balochistan, Quetta. A significant reduction in plant length, cover, number of leaves and total chlorophyll contents for V. *vinifera* L. was observed. The maximum reduction of all **investigated variables such as plant length, cover, number of leaves and total chlorophyll contents on the fourth week of observation conform that long time accumulation of road side dust put long term savior effects on plant growth and its pigmentations. The results of this study also reveal that there is negative correlation between the amount of dust accumulation and plant growth parameters, as the amount of dust increased plant growth decreased, respectively.**

Key words: Dust, seedling growth, plant length, cover, number of leaves, and photosynthetic pigments.

INTRODUCTION

It is difficult to estimate the effects of air pollutants because the organisms are exposed to wide range of uncontrolled variables (parasites, weather conditions and complex mixture of pollutants). On the physiological and morphological point of view, the plants from polluted sites present important changes especially regarding their colors, shapes, leaf length, width, area and petiole length. However, despite these changes, plants survive well at the polluted environment (Leghari and Zaidi, 2013). Leaf is the most sensitive part to be affected by air pollutants. Therefore, the leaf at its various stages of development, serves as a good indicator to air pollutants. Air pollutants can directly affect plants via leaves or indirectly via soil acidification (Steubing et al., 1989). Pollutants when absorbed by the leaves cause a reduction in the concentration of photosynthetic pigment viz. chlorophyll which directly affects the plant productivity. Plants are the only living organisms, which have to suffer a lot from automobile exhaust pollution because they remain static at their habitat. Properties of both particulate matter and the vegetation are important in deciding their interactions, and consequently the effectiveness of particle removal from atmosphere. A number of recent studies observed that in urban atmospheres the concentrations of PM_{10}

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and $PM_{2.5}$ airborne aerosols show good agreement with traffic-related pollutants and other combustion processes (Prajapati and Tripathi, 2007); whereas, crustal material, re-suspended road dust and long-range transport events are mainly identified as sources of the coarse particles (Park and Kim, 2005; Vallius et al., 2005). Vehicular emissions and agricultural activities generate local dust concentrations close to the source which exceed environmental guideline values (Leys et al., 1998; Manins et al., 2001). Heavy metals released from automobiles are extremely toxic metal and reduces plant growth and morphological parameters. Therefore, the study conducted by Ahmad et al. (2012) is in agreement with this study stating that the cadmium had toxicity at 5 mgL^{-1} in case of root and shoot growth. Air pollution due to vehicular emission mostly arises from cars, buses, minibuses, wagons, rickshaws, motorcycles and trucks. These resources introduce varieties of pollutants (oxides of nitrogen and sulphur, hydrocarbon, ozone, particulate matters, hydrogen fluoride, peroxyacyl nitrates, etc.) into the environment which not only put adverse effect on the health of human beings, and animals, but seriously threating the trees and crops of such areas. Research studies revealed that plants growing in the urban areas are affected greatly by these pollutants (Uaboi-Egbenni et al., 2009).

Now it is necessary to identify some principles that may indicate these impacts, and the need for mitigation measures (Prajapati and Tripathi, 2008a, d). Air pollution in Quetta city (study area) is rising to an alarming state rapidly since the last few decades due to heavy automobile activities. Rapid increase in automobile activities and traffic congestion contributes most of air pollution problems, resulting in damage to the plants growth. Therefore, the present work was mainly designed to analyze the effects of air born-dust pollution, dom-nantly presented by automobile, industrial pollution and microclimate on physiology and morphology of *Vitis vinifera* L. (grape), because grape is one of the most widely grown fruit crops in the investigated area (Quetta city). They have a wide distribution, which indicates a high economically, ecologically plasticity in study area. Our goal in the present study was to evaluate the relationships between airborne dust deposition, physiological and growth parameters of *V. vinifera* L. (grape).

MATERIALS AND METHODS

Description of study area

Quetta is the provincial capital of Balochistan province. It is situated at an elevation of 1676 to 1900 m above sea level. The climate of the area is generally dry and cold. Maximum rainfall and snowfall occurs in January and February. Summer season is moderate, while June and July is the hottest month with maximum temperature of 30 and 20°C, respectively. January is the coldest month with mean maximum and minimum temperature of about 11 and -3°C, respectively (Anonymous, 1998). This variation in climate is the main ecological factor, due to which vegetation of southern zone differs from that of North. Due to variation in these factors, there is a variation in the penology of the vegetation from season to season.

The experiment was conducted in green house under the uniform natural environmental conditions at the Campus University of Balochistan, Quetta during March and April, 2012 in pots. Healthy and uniform size seeds of *V. vinifera* L. were collected from the University Campus. The seeds were sown in garden soil at 1 cm depth in large pots and watered regularly. After two weeks of their germination, uniform sized seedlings were transplanted in pots of 25 cm in diameter and 22 cm in depth containing the same garden soil in which they were germinated (Kabir et al., 2009). Road side dust was applied with ratio 1, 2, 3, 4 and 5 g, respectively on the aerial parts of each plant twice a week, except the control (0). The height of plant and cover were measured with a measuring tape. The numbers of leaves were also counted every week. The total experiments lasted six weeks (Mishra and Pandey, 2011).

Photosynthetic pigments analysis

Three grams of fresh leaves were put in 100% acetone (50 ml for each gram), homogenized (homogenizer B. Braun Melsungen, Germany) at 1000 rpm for 1 min. The homogenate was then filtered through double layered cheese cloths and centrifuged at 2500 rpm for 10 min. The extract was removed to a 10 ml graduated cylinder using a Pasteur pipette. An aliquot of the clear leaf extract (supernatant) was transferred with a pipette to a 1 cm path length cuvette and absorbance readings taken against a solvent blank in a ultra violet- visible (UV-VIS) spectrophotometer at 663, 645, 470, 435 and 415 nm wavelength to determine the concentrations of photosynthetic pigments like chlorophyll-a, chlorophyll-b and total chlorophyll content (TCh) using the formula given by Lichtenthaler (1987). The ratio of absorbance 663 to 645 nm are the parameter for maximum chlorophyll (a and b) absorbance in the experiment (Ronen and Galun, 1984).

Quantification of pigments (For 100% acetone)

Chl-a =12.7DX663 - 2.69DX645 x V/1000W (μg g^{-1} f.wt.)
Chl-b =22.9DX645 - 4.68DX663 x V/1000W (μg g^{-1} f.wt.)
TCh = Chlorophyll a + b (μg g^{-1} f.wt.)

Where, DX = Absorbance of the extract at the wavelength x nm, V = total volume of the chlorophyll solution (ml), and W = weight of the tissue extract (g).

Statistical analysis

The standard deviation values of the means were calculated for comparison of site categories. To determine the significance of the samples, paired t-test was performed (Steel and Torrie, 1980).

RESULTS

Effect of road side dust application on *V. vinifera* L. length, number of leaves, plant cover, total chlorophyll contents, over all comparison between plant variables and correlation between amount of dust applied and plant growth are shown in Tables 1 to 5 and Figure 1. In

Table 1. Effect of road side dust (gm) application on plant length during some weeks of growth.

Dust applied (gm)	Plant length (cm)			
	Weeks after dust application			
	1	2	3	4
Control	10.58	12.63	13.55	15.12
1	6.82	7.42	7.85	8.50
2	6.50	7.05	7.4	8.12
3	5.62	6.00	6.75	7.52
4	4.65	5.11	6.00	6.50
5	4.60	5.05	5.87	6.36
Average	5.6 (1.0)	6.1 (1.1)	6.8 (0.9)	7.2 (3.3)
Decrease percentage in Plant length with respect to the control	87.6	106.1	106.1	109.4
Confidence level (T-test)	**	***	***	***

Values in parenthesis indicates standard deviation, **highly significant p< 0.01, *** very highly significant p < 0.001.

Table 2. Effect of road side dust application on number of leaves.

Dust applied	Number of leaves			
	Weeks after dust application			
	1	2	3	4
Control	11.5	13.4	22.4	31.6
1	9	11	14	18
2	9	10	12	16
3	7	8	10	13
4	6	7	9	11
5	6	7	8	10
Average	7.4 (1.5)	8.6 (1.8)	10.6 (2.4)	13.6 (3.4)
Decrease percentage in No of plant leaves with respect to the control	55.4	55.8	111.3	132.4
Confidence level (T-test)	*	*	***	***

Values in parenthesis indicates standard deviation, *slightly significant p < 0.05, **highly significant p< 0.01, *** very highly significant p < 0.001.

general, *V. vinifera* L. showed a significant decrease in its growth, number of leaves, plant cover and total chlorophyll content due to road side dust treatment. Results also indicate that there was negative correlation between the application of dust concentration and growth rate during all the four weeks of investigation. As the concentration of dust application increased all the investigated parameters decreased, respectively. The study also reveales that reduction in plant length, plant cover, number of leaves and total chlorophyll contents varies from week to week and variation was also noted due to different concentration (1 to 5 g) of dust applied.

The maximum and minimum average plant length (7.2 and 5.6 cm) was noticed during 4th weeks and 1st week of dust application, respectively. Decrease percentage in plant length due to dust applied with respect to the control ranging from 87.6 to 109.4% and statistical analysis using T-test revealed that there was slightly to high variation in plant length between control and dust treatment (Table 1).

The numbers of leaves were significantly decreased in *V. vinifera* L. together with increasing dust accumulation in plant. The maximum and minimum average number of leaves (13.6 and 7.4) was noticed during 4th weeks and 1st week of dust application, respectively, on the other hand the plant with control site had 11.5 to 31.6 numbers of leaves. Decrease percentage in plant leaves due to dust applied with respect to the control ranging from 55.4 to 132.4% and statistical analysis using T-test indicated that there was slightly to high significant variation in number of leaves between control and dust treatment plants (Table 2). Table 3 shows that the average plant covers at dust application site were in the range of 93.7 to 134.0 cm^2, while plant at control site was 173.9 to

Table 3. Effect of road side dust application on plant cover (cm^2).

Dust applied	Plant cover			
	Weeks after dust application			
	1	2	3	4
Control	173.9	203.4	230.4	280.6
1	116.0	133.2	156.2	170.3
2	100.2	119.2	121.1	153.2
3	92.3	105.4	115.2	121.1
4	80.1	93.1	100.2	113.0
5	80.1	93.0	100.1	112.2
Average	93.7 (15.1)	108.8 (17.4)	118.6 (23.0)	134.0 (26.3)
Decrease percentage in plant cover with respect to the control	85.6	87.0	94.3	109.5
Confidence level (T-test)	**	**	**	***

Values in parenthesis indicates standard deviation, **highly significant p< 0.01, *** very highly significant p < 0.001.

Table 4. Effect of road side dust application on total chlorophyll contents.

Dust applied (gm)	Total chlorophyllcontents (µg g^{-1} f.wt.)			
	Weeks after dust application			
	1	2	3	4
Control	58.1	61.3	66.2	68.3
1	51.4	54.3	55.3	57.2
2	45.2	48.0	51.2	53.2
3	44.4	45.3	48.6	50.4
4	40.2	42.4	44.2	45.5
5	35.1	38.3	40.2	40.6
Average	43.3 (6.1)	45.6 (6.0)	47.9 (5.9)	49.4 (6.5)
Decrease percentage in total chlorophyll contents with respect to the control	34.4	36.8	38.2	38.4
Significance level (T-test)	n.s	n.s	n.s	n.s

Values in parenthesis indicates standard deviation, n.s: not-slightly significant p < 0.05.

Table 5. Correlation b/t dust applied and plant growth.

Plant variable	Correlation			
	Weeks after dust applied			
	1	2	3	4
Plant length (cm)	-0.94	-0.96	-0.98	-0.99
Number of leaves	-0.96	-0.95	-0.94	-0.90
Plant Cover (cm^2)	-0.96	-0.95	-0.94	-0.90
Total chlorophyll contents (µg g^{-1} f.wt.)	-0.98	-0.99	-0.99	-0.99

280.6 cm^2 which showed slightly to high significance. The decrease percentage in plant cover was noted to be 85.6 to 109.5%. Dust accumulation altered the chlorophyll and carotenoid contents in *V. vinifera* L. Greater decrease in total chlorophyll contents was clearly observed at 38.2 and 38.4% in 3rd and 4th weeks of dust application, respectively. Statistical analysis using T-test showed non-significant variation in total chlorophyll contents between control and dust treatment plants but there was variation within the dust treatment plants (Table 4). The plant growth (all the investigated variables) gradually decreased with the increased dust concentration. There was negative correlation between dust accumulation and plant growth as shown in Table 5. The minimum growth was noticed in the plants treated with 4 to 5 g dust without significant change between plants treated with 4

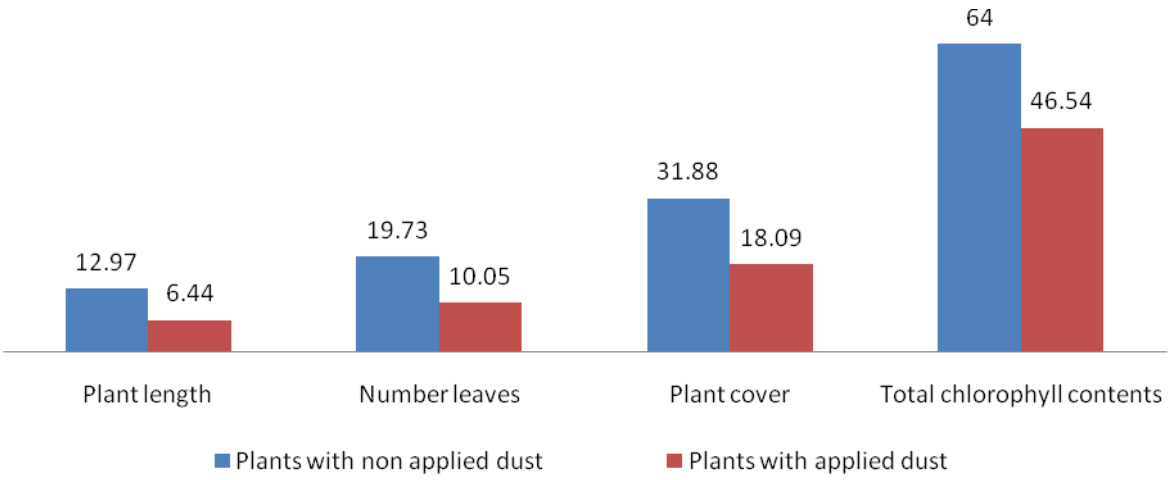

Figure 1. Over all comparison b/t different plant parameters of plants with non applied dust and plants with applied dust.

to 5 g during all the weeks. On the other hand, except total chlorophyll contents all the other investigated parameters showed significant over all difference between polluted and control site plants as indicated in Figure 1.

DISCUSSION

Road side dust had a significant effect on the growth of *V. vinifera* L. compared with non-dusted plant (*V. vinifera* L.). Reduction in plant height, cover, number of leaves and total chlorophyll contents of *V. vinifera* L. showed that the losses are generally attributed to the road side dust which contained mixture of toxic metals. The results obtained are in close conformity with those reported by Prajapati (2012) and Stratmann (1966), who dusted plants with dust ranging from 1 to 48 g/m^2 day^{-1} and concluded that dust falling on the soil caused a shift in pH to the alkaline side, which was unfavorable to oats but favorable to pasture grass.

Reduction of plant length might be due to the decrease in phytomass, net primary production and chlorophyll content in response to the road side dusts, similar to observation also noted by Prasad and Inamdar (1990) in Vignamungo (Black gram) and Armbrust (1986). A significant reduction in plant cover of *V. vinifera* L. suggests that it is more sensitive to road side dust. The observation noted by Shafiq and Iqbal (1987) is in agreement with these results; they found a reduction in the number of species around the heavily polluted cement industrial units in Karachi. Darley et al. (1966) demonstrated that dust deposited on bean leaves in the presence of free moisture interfered with the rate of carbon dioxide exchange, but no measurements of starch were made. The decreased in chlorophyll contents in the leaves of *V. vinifera* L. might be due to long time accumulation of dust on plant leaves that interrupt the sun light to reach the leaf. Prajapati and Tripathi (2008) observed reduction of pigment content in plant species due to dust accumulation. Similar results were also reported by Prasad and Inamdar (1990), they found that the dust kiln showed a reduction in chlorophyll content, protein, starch, yield and phytomass in ground nuts (*Arachis hypogaea* L.). A significant reduction in chlorophyll content, photosynthesis and growth in cotton due to particulates (dust) was also reported by Armbrust (1986). A significant reduction in leaf number for *V. vinifera* L. agrees with the findings of Laghari and Zaidi (2013); Anda (1986). Studies of biochemical changes and pollution effects on the plant metabolism, that is, reduction in chlorophyll and completely closed stomates (Ahmed and Qadir, 1975) revealed that these parameters are important in regulating the productivity and also the number of flowers and seeds produced.

Conclusion

On the basis of this study, it could be concluded that the phenological behavior of *V. vinifera* L. was found to be highly affected. It is clear that the road side dust pollution is an operative ecological factor causing deterioration in the quality of our environment and economic crops. It is suggested that the highly dust tolerant local plant species (*Pinus halepensis* (Miller.) and *Eucalyptus tereticornis* L.) should be planted around the road side. It is also suggested that complete analysis of road side dust containing toxic pollutants should be carried out in detail. The experimental analysis of the effect of dust on vegetation helps to recommend plants for use as screens or green belts in urban areas in order to mitigate dust and

improve air quality (Yunus et al., 1985).

Conflict of interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Improving oil biodegradability of aliphatic crude oil fraction by bacteria from oil polluted water

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Water samples were collected from three oil polluted stations, two replicates for each station, from southern region of Shatt Al-Arab estuary, and southern of Basrah city during the period from September to October 2011. The mineral salts medium was used to isolating oil biodegrading bacteria. Four bacterial species were identified according to their morphological and biochemical profiles as: *Aeromonas hydrophila*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. The percentage of biodegrading ability of *B. subtilis* and the mixture of these bacteria to n-alkanes and isoprenoids (pristine, phytane) were measured and compared with control. Crude oil is used as a sole source of energy and the incubation period was 24 days, the hydrocarbons loss are detected each 6 day interval using capillary gas chromatography. Bacterial species were exposed to biological mutation by using Maillard reactions to improve the n-alkanes and isoprenoids (pristine, phytane) biodegradability. For this, a mixture of glucose-lysine in a concentration of 4 M was used to mutate *B. subtilis* and *A. hydrophila* while for *P. aeruginosa* and *P. fluorescens* a mixture of glucose-arginine in a concentration of 9 M. Biodegradability percentage was increased for *B. subtilis* from 60.6 to 92.5% and ranged from 37 to 72.3% for the other species. Also the bacterial mixture biodegradability for oil increased from 78 to 87.5%.

Key words: Oil biodegradation, *Aeromonas hydrophila*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, n-alkanes, Maillard reactions.

INTRODUCTION

The hydrocarbons and petroleum constitute one of the main environmental pollutants. The petroleum contains thousands of individual hydrocarbons and related compounds. Their main components are saturated (n- and branched-chain alkanes and cycloparaffins rings), aromatic and polynuclear compounds (PAHs) and resins and asphaltenes (Rosa et al., 2006).

Bioremediation of the environment polluted by crude oil relies on the fact that indigenous microbial population can biodegrade most of the hydrocarbons present in oils, mineralizing them into carbon dioxide and water (Fritsche and Hofrichter, 2008).

It is uncommon to find organisms that could effectively degrade both aliphatic and aromatics possibly due to differences in metabolic routes and pathways for the degradation of the two classes of hydrocarbons (Fritsche and Hofrichter, 2008). However, some reports have suggested the possibility of bacterial species with propensities fordegradation of both aliphatic and aromatic hydrocarbons simultaneously (Salam et al., 2011).

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Figure 1. The three oil polluted locations (Sampling stations 1, 2 and 3) from southern part of Shatt Al-Arab estuary, southern of Iraq.

Applications of genetically engineered microorganisms (GEMs) in bioremediation have received a great deal of attention to improve the degradation of hazardous wastes under laboratory conditions. The genetically engineered bacteria showed higher degrading capacity. However, ecological and environmental concerns and regulatory constraints are major obstacles for testing GEMs in the field. These problems must be solved before GEM can provide an effective clean-up process at lower cost (Das and Chandran, 2011). The heat-induced reaction of amino groups of amino acids, peptides, and proteins with carbonyl groups of reducing sugars such as glucose, results in the concurrent formation of so-called Maillard reaction products (MRPs) (Friedman and Mottram, 2005).

The aim of this study at first step was to isolate local bacterium (bacteria) which has the ability to degrade crude oil (n- alkanes) individually or in mixed culture. The second step is improving their abilities for degradability after mutating them by using of Maillard reaction products as mutagens.

The exposure of organisms to ultraviolet light or treated with nitrous acid has been employed with relative successes. Such mutants, under optimal growth conditions, could possess enhanced petroleum degradation potentials than their parents (Idise et al., 2010).

MATERIALS AND METHODS

Water sampling

Water samples were collected from three oil polluted locations from southern part of Shatt Al-Arab estuary, southern of Iraq (Figure.1).

Water samples were collected in sterile 500 ml glass bottles. The samples were placed on ice until returned to the laboratory.

Isolation and identification of bacterial species

1 ml of each sample was cultured in a conical flask containing 100 ml mineral salt medium (MSM), the composition of the medium was: 0.3 g KCl, 1.0 g K_2HPO_4, 0.5 g KH_2PO_4, 0.5 g $MnSO_4.7H_2O$, 0.2 g $CaCl_2.2H_2O$, 30 g NaCl, and 0.01 g $FeSO_4.7H_2O$ (Fujisawa and Murakami,1980) with 0.1 ml crude oil (Provided from Al-Shua'aba Refinery) and 1000 ml H_2O. Decimal dilution of 7-21 days grown culture was cultivated at 30ºC for 24 h. Pseudomonas agar base (Himedia) was used to isolate *Pseudomonas* species, Ampicillin Dextrin agar (Himedia) was used to isolate *A. hydrophila* and Luria-Bertani agar (Himedia) was used to isolate *B. subtilis*.

Presumptive colonies were subcultured on nutrient agar and were subjected to Gram stain, oxidase, catalase, nitrate reduction, motility, gelatin liquefaction, endospore, indole, methyl red , VogesProskauer, citrate utilization, growth at 4ºC, growth at 41ºC, fluorescens and H_2S tests.

Degradability study of crude oil

1 ml of *B. subtilis* broth culture as well as the bacterial mixture was incubated separately in 250 ml Erlenmeyer flasks containing 50 ml of MSM at 20 FID C for 24 days with shaking at 120 rpm using cooling incubator shaker (GermanySartorius Stidem-Certomat). All the experiments were carried out in four duplicates, each flask was taken out from the incubator in 6 days intervals for estimation of residual crude oil.

Extraction of residual crude oil

Residual crude oil was extracted by liquid-liquid extraction as described by Adebusoye et al. (2007). After removing the aqueous phase with separating funnel, the residual oil was dried in the oven at 40ºC to remove CCl_4.

The aliphatic fraction was separated by using separation column (25 cm length, 3 cm diameter) containing 8 g silica gel over a little amount of wool cotton (Farid, 2006). The residual oil dissolved with 25 ml of n-hexane and poured in the separation column and drawn off the aliphatic fraction in 50 ml beaker. Control flasks were also extracted similarly, n-alkanes hydrocarbons were estimated by FID gas chromatography (Agilent Chem Station).

Maillard reaction products (MRPs)

Four mixtures of amino acid and reduced monosaccharide were prepared: (glucose+lysine; glucose+arginine; fructose+lysine and fructose+arginine) in different concentrations from 0.1 to 10 M (Kitts et al., 1993). Equals volumes of amino acids and sugars were heated (after adjusting pH to 9) by autoclaving at 121°C for 1 h, the final brown products used to induce bacterial mutations.

Mutagenesis of isolates using MRPs

Mutant was initiated by using the method reported by Defontaine et al. (1999), pure isolates of each bacterial type was cultivated on Brain Heart agar by spreading method. Then 1 cm diameter well was made in the center of each culture medium filled with 0.1 ml of MRPs then incubated at 35ºC for 24 h, the colonies appear in the inhibition zone around the wells considered as mutants. Degradation study was repeated again by using mutant strains as described previously to distinguish the changes in the biodegradability after and before mutation.

RESULTS

The effective mixture of MRPs (glucose+Lysine) is at the concentration 4 M which lead to induced a random mutation in *B. subtilis* and *A. hydrophila* while the effective mixture to *P. aeruginosa* and *P. fluorescens* was (glucose-arginine) at the concentration 9 M. The concentrations of n-alkanes (C9-C28) with isoprenoid-spristine and phytane were calculated by comparing with standards solutions. Figure 2 shows gas chromatography results of n-alkanes of control sample. Biodegradation of n-alkanes by bacterial mixture at zero day, before and after mutation was shown in the results of gas chromatography (Figure 3). The biodegradation percentage was improved from 78 to 87.5 % (Table 1).

Figure 4 Shows the gas chromatography results of n-alkanes of crude oil incubated with *B. subtilis* at zero day, before and after mutation.The biodegradation percentage was improved from 60 to 92.5% (Table1). Gas chromatography results of n-alkanes of crude oil for days 6 and 18 were not reported.

DISCUSSION

Crude oil biodegrading microorganisms which are able to utilize crude oil as a sole source of energy distribute widely in different environments; air, water and soil (Magot, 2005). In the present study, four bacterial species have been identified according to morphological and biochemical profile in accordance with Holt et al. (1994) and De Vos et al. (2009). They were capable of oil degradation as a source of carbon that matches with many studies (Hamzah et al., 2010; Uğur et al., 2012; Malik and Ahmed, 2012). The results show that the bacteria degrading in the beginning, the lower and higher hydrocarbon chains while the middle chains were degraded later and these results are in accordance with Bello (2007).

Mineral salts medium has been used in many studies (Farid, 2006; Del'Arco et al., 1999; Salam et al., 2011) in spite of simple variations in its composition and concentrations, such as the addition of nutrients and biostimulators for enzymes which are involved in the biodegradation mechanisms.

Adding crude oil as a sole source of carbon is not sufficient because, microorganisms need another nutrients so that nitrogen and phosphorus can been added in high concentrations to the medium, but sulfur, iron, magnesium and sodium are added in low concentrations, Atlas(1984). Oil layer phenotypic changes in the test flasks were due to the growth of the studied isolates and this confirms the ability of bacteria to utilize crude oil and transform it to small droplets. This is evidence that

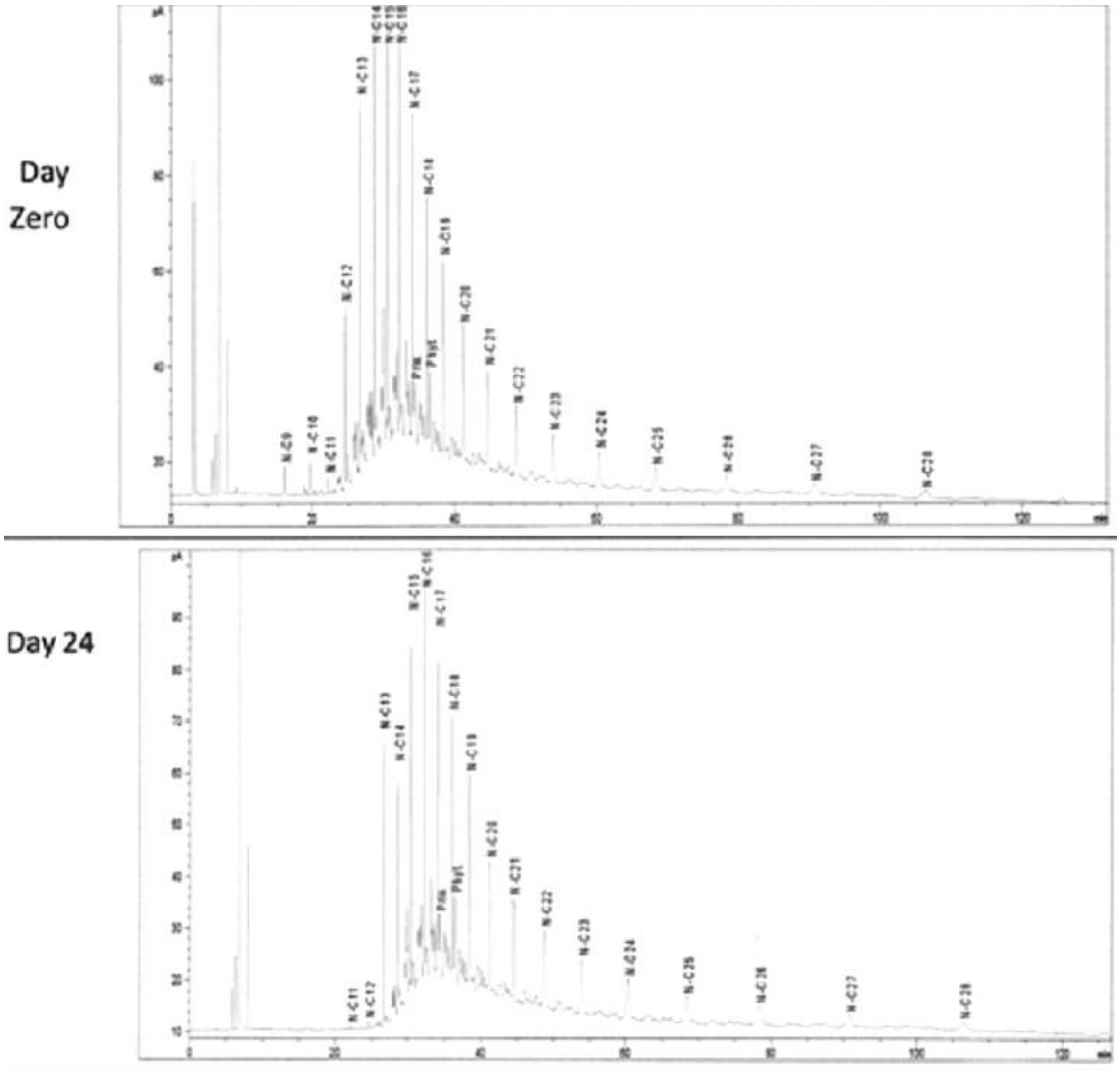

Figure 2. Capillary gas chromatography results of n-alkanes of control flasks at day zero and day 24.

the isolates emulsified the crude oil due to bioemulsifiers which are produced by oil degrading bacteria in the test flasks and no changes in the control flasks that match with Naser (2000).

The results of gas chromatography showed that the n-alkanes with short chains (C9-C12) are most degradable in early stages of incubation while longer chains (C14-C26) were degraded in the later stages. Malik and Ahmed (2012) suggested that the compounds with low molecular weights (C8-C12) dis-appear even in control flasks as a result of evaporation.

Heated food systems contain hundreds of chemical compounds, some being mutagenic, for example: reductones, dicarbonyles, pyrozines and furan (Powrie et al., 1986). The heat-induced reaction of amino groups of amino acids, peptides, and proteins with carbonyl groups of reducing sugars such as glucose results in the concurrent formation of so-called Maillard browning products or melanoidins (Friedman and Mottram, 2005). In this study, Maillard reaction products were used as mutagenic agents for the bacterial types to improve the ability of this bacterium to utilize aliphatic fraction of crude oil.

As a results of the great diversity of these products , each one affects in a different way on DNA, for example, phenols remove purines especially guanine so that site will be empty and there are four chances to link opposite nitrogen base of DNA during multiplication that leads to a change in nitrogen base sequence.Change in the genetic code resulting in a change in the nature of the formed enzymes (Al-Dalali,1994).

Mutation improved biodegradabilityof *B. subtilis* about 32% that leads to raised bacterial mixture biodegradability 9.5% (Table1) with statistically significant differences. These positive changes in bacterial ability may be induced due to formation of new enzymes involved in this process or increment in the enzymes activity. Testing this mutant strain in the field may show good possibility for using it in bioremediation later on.

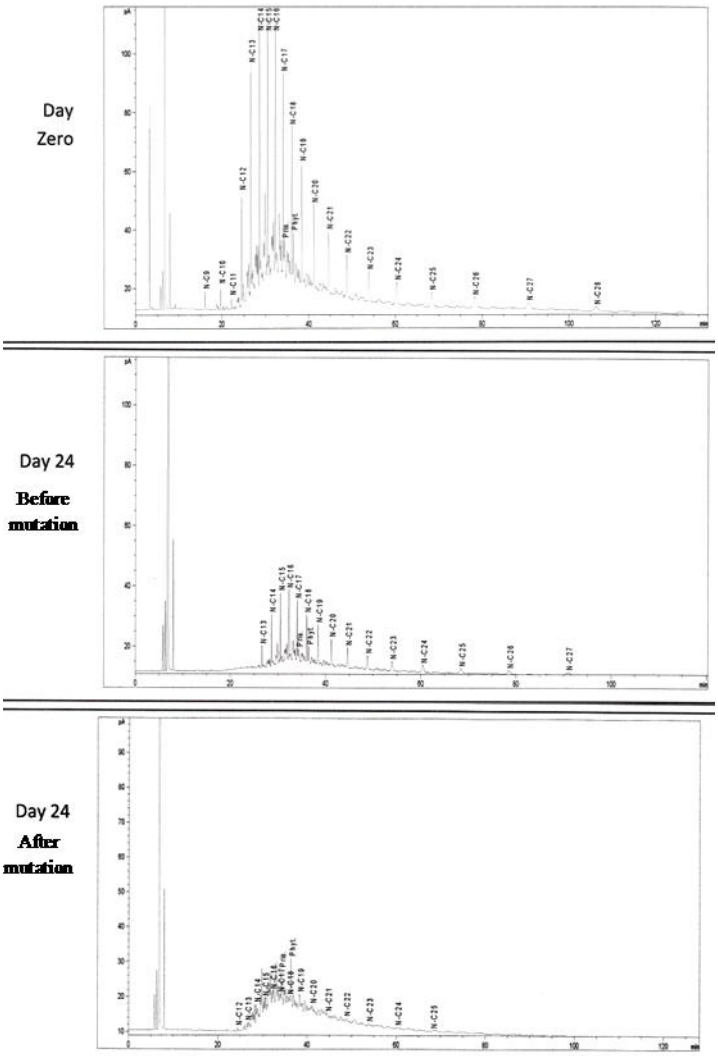

Figure 3. Capillary gas chromatography results of n-alkanes of bacterial mixture at day zero, day 24 before mutation and day 24 after mutation.

Table 1. Biodegradation percentage (BD %) of Bacillus subtilis and mixture before and after mutation

Day	Before mutation (BD%)	After mutation (BD%)
Bacterial mixture		
Zero	0	0
day 6	48.81	14.77
day 12	65.59	24.49
day 18	71.64	84.17
day 24	78	87.5
Bacillus subtilis		
Zero	0	0
day 6	16.76	46.6
day 12	20.9	63.58
day 18	49.64	72.13
day 24	60.6	92.5

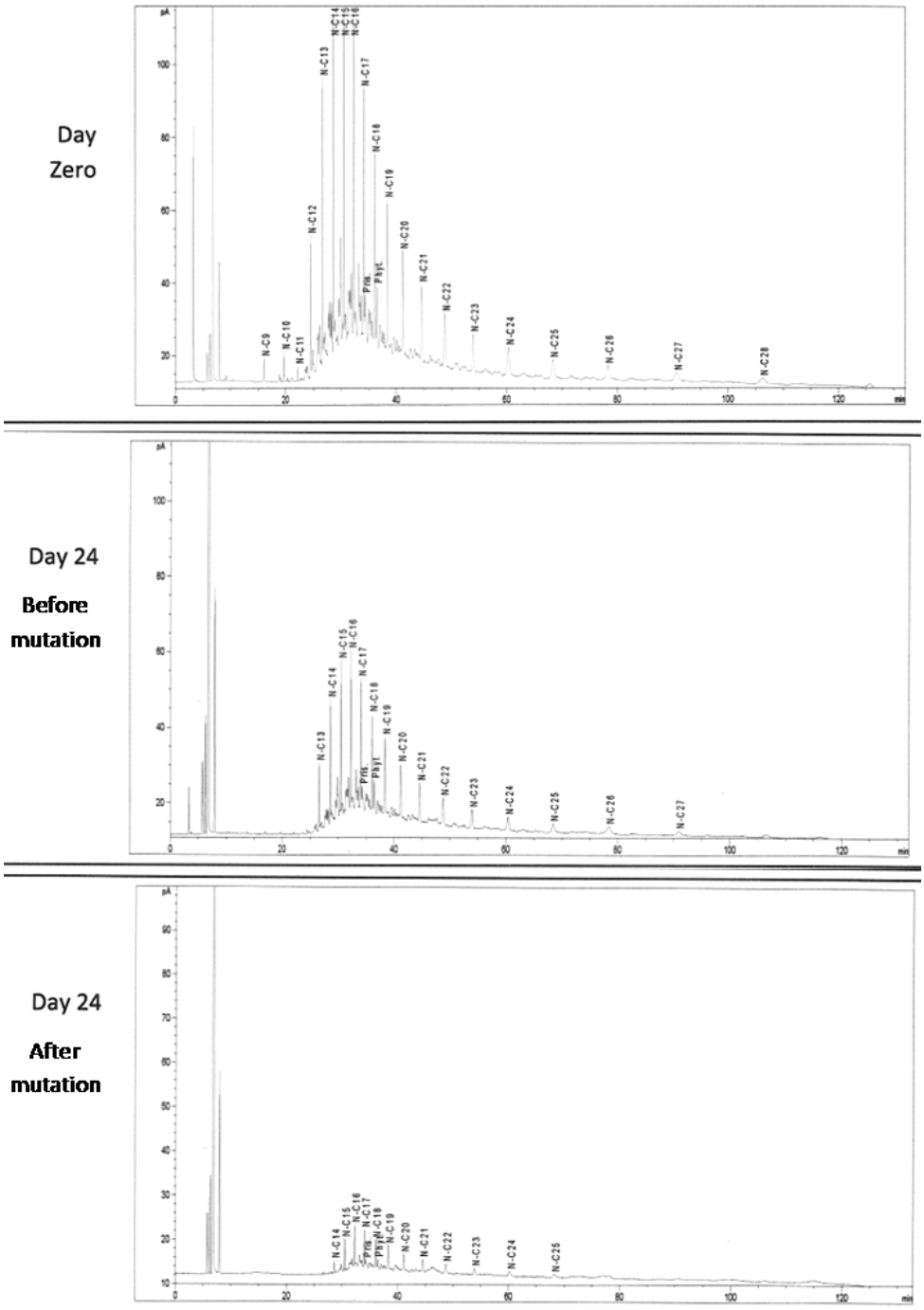

Figure 4. Capillary gas chromatography results of n-alkanes of Bacillus subtilis at day zero, day 24 before and after mutation.

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Yield of maize (*Manoma spp*) affected by automobile oil waste and compost manure

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The study is aimed at evaluating the effects of compost manure on the remediation of automobile oil waste polluted soils, and on the yield parameters (plant height, leave surface and dry matter weight) of maize (*Manoma spp*). Analyses of soil samples of contaminated and uncontaminated sites collected with core samplers at 10 cm depth and auger samplers at two depths (surface, 0 – 20 cm; subsurface, 20 – 40 cm) were examined for chemical and physical properties, including poly aromatic hydrocarbons. Polluted soils were biotreated for testing maize in a greenhouse. Soil physical and chemical properties decreased with depth and were significantly (P<0.05) affected by contamination. Decreases in soil poly aromatic carbon from original concentration were observed. Phyto-assessment showed that maize seedlings bio-accumulated heavy metals in polluted soils, which made their survival rate marginal relative to those grown in uncontaminated soils. Soil amendment with compost manure significantly (P<0.05) improved soil properties and maize yield variables. Ecological risk factor (HQ>1) of heavy metals (for example; Zn, Cu, Mn) was high for maize cultivation. Paradigm approach emphasizing sustainable biological soil systems management is desired. Particularly, bioremediation of oil polluted soils using organic materials, and siting of mechanic villages several kilometers away from major land uses (residential houses, farm lands, and usable water bodies) are important for protecting the soil resources for agricultural purposes, and to ensuring environmental sanity and sustainability.

Key words: Automobile oil wastes, phytotoxicity, soil amendment, poly aromatic carbons.

INTRODUCTION

Petroleum hydrocarbon, its refined products and byproducts plays important role in the development of the economy of nations, including Nigeria. Refined products such as petrol, diesel, kerosene, among others and byproducts like oil, waxes drives the economy as it creates livelihoods and development.

However, in routine maintenance of vehicles, mechanic operators would always discharge petroleum and its derivatives (example, spent engine oil) leaving behind serious environmental concerns. Arising from indiscriminate location of mechanic sites and the effects of soil and environmental contamination by the petroleum byproducts in most cities of Enugu State, Nigeria. The government decided to concentrate these motor vehicle

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servicing centers within a location and designate it as "mechanic village".

A mechanic village is an area of open land allocated to automobile workers in the vicinity of urban/peri-urban areas. In these places, they specialize in repairs and servicing of vehicles. They also serve as large scale skill acquisition centers for auto mechanics, auto electricians, spray painters, panel beaters, welders, spare parts fabricating and machine operators, spare parts dealers among others.

Even though heavy metals are naturally present in soils, but the environment of auto workshops is increasingly exposed to changes due to anthropogenic sources that could create vast amount of various wastes and pollutants, and adequate attention has not been given to its disposal. These pollutants could be drastic and as such affect ecosystem substantially. It also can affect the trophic chain, plants, animals, and man and can remain in the soil for a long time (Osuji and Nwoye, 2007).

The disposal of spent engine oil (SEO, a brown-to-black liquid) into gutters, water drains, open vacant plots and farm soils is a common practice especially by motor mechanics. This waste engine oil is usually obtained after servicing and subsequently draining from automobile and generator engines (Anoliefo and Edegbai, 2000).

Relatively large amounts of hydrocarbons in the used oil, including the highly toxic polycyclic aromatic hydrocarbons have been reported (Wang et al., 2000). Shayler et al. (2009) report showed that engine oil may contain chromium, lead, molybdenum, or nickel from engine wear, while used batteries release lead or mercury. These heavy metals may be retained in soils in the form of oxides, hydroxides, carbonates, exchangeable cations, and/or bound to organic matter in the soil.

Spent engine oil hydrocarbons, when present in the soil, creates an unsatisfactory condition for life in the soil, thus causing poor soil aeration, immobilization of soil nutrients, and lowering of soil pH (Atuanya, 1987), unsatisfactory for plant growth due to the reduction in the level of available plant nutrient (soil bio-physical and chemical properties) or a rise in toxic levels of certain elements such as iron and zinc (Udo and Fayemi, 1975).

Due to inadequate waste disposal infrastructure, proper management of these wastes has become a cause for concern. The processes that lead to the removal of these heavy metals and hydrocarbon pollutants from the environments involve physical, chemical and biological alternatives. Madsen (1991) has shown that biological approach is most preferred to physical and chemical alternatives because they reintroduce the contaminants into the environment.

Bioremediation is the spontaneous process in which biology, especially microbial catalysis, acts on pollutant compounds, thereby eliminating environmental contamination (Madsen, 1991). Maize is a staple food crop that provides protein and calories for human need. It can easily adapt in famers' cropping system with low input technology of production.

The effects of remediation strategies on the phyto-toxicity of waste crude oil-polluted soil on maize yield parameters constitute an important thrust of this study. Studies, elsewhere, have obtained stabilization of waste through biotreatment (Diaz et al., 1996) but Enugu state has received less attention in these areas of assessment. Also, with paucity of data upon which to determine soil health for a specific land use type, especially in study locations, many fundamental questions concerning the mechanic effluent fluxes and distribution of soil nutrients in relation to maize crop production remain unanswered.

The study aims to provide information on the effects of compost manure on the remediation of automobile oil waste polluted soils, and on the yield parameters (plant height, leave surface and dry matter weight) of maize (*Manoma* spp).

MATERIALS AND METHODS

Description of study area

The study was conducted in the screen house of the University of Nigeria, Nsukka using oil waste contaminated and uncontaminated soils collected from automobile workshops located in Udi, Nsukka, and Udenu local government areas (LGAs) in Enugu State, Nigeria.

Udi LGA covers an area of 897 km^2 and lays 6°19'N and 7°26'E with a population of about 234, 002. Nsukka LGA lies by latitude 6°51'24" and longitude 7°23'45"E with land area of 45.38 km^2. Udenu LGA lies between coordinates 6°55'N and 7°31'E with a total land area of 897 km^2 and a population of about 178, 466 (http://www.nipost.gov.ng/postcode.aspx).

The vegetation of Enugu state is of the semitropical rainforest type and complemented by typical grassy vegetation. The soils are ferrallitic (also called red earth or acid sands) ultisol. The state is predominantly rural agrarians, with a substantial proportion of its working population engaged in farming.

Field work

Soils contaminated with automobile waste oil were collected from Ngwo, Umuakashi and Obollo-Afor mechanic villages situated, respectively, in Udi, Nsukka and Udenu LGAs within South eastern Nigeria. The soils were sampled at depth of 0-20 and 20-40 cm with soil auger within an area measuring 50 × 50 m using the random sampling technique. Uncontaminated soils were collected for standardization. Core ring (volume - 96.6 cm^3) samples were taken at soil depth of 0-10 cm. All soil samples were used for soil property determinations and maize planting in the screen house.

Screenhouse studies

The soils collected from the fields of each location weighing 5 kg were air dried. Thereafter 2.5 kg of each site soil was measured into poly bags. Each poly bag was amended with 1.5 kg of air-dried compost manure (CM) except the control. Treatment details were as follows:

i) Three bags each of contaminated and uncontaminated soil

samples with 0 kg CM
ii) Three bags each of contaminated and uncontaminated soil samples with + 1.5 kg CM.
The compost manure was thoroughly mixed into the soils and 0.5 kg of river sand added. The entire set up was left to decompose for three months period. 50 ml of water was applied at intervals.

Phyto assessment

After the 3 months period, soil in each bag was turned and properly mixed and watered (200 ml) prior to maize sowing. *Manoma spp* of improved maize seeds were sown into each bag at the rate of 3 seeds to a depth of 3 cm, and thinned down to 2 seedlings per bag after seedling had attained 2-leaf stage. The soil bags were constantly weeded. Measurements were made on the growth and yield parameters (height, leaf surface and dry matter yield) of the improved maize.

Laboratory analysis

Bulk density (Bd) and saturated hydraulic conductivity (Ksat) were determined using Grossman and Keinch (2002) method, while total porosity was calculated using Bd data. The auger soil samples were air-dried in the laboratory ground and passed through a 2 mm sieve. Sieved samples < 2 mm soil fraction was bagged for routine analysis.

The fraction of sand, silt, and clay was determined using hydrometer method (Gee and Or, 2002) with NaOH as dispersant. Soil pH was determined by McLean (1982) method. Total nitrogen was determined using micro- Kjeldahl (Bremner and Mulvaney, 1982) method. Soil organic carbon was Soil measured by combustion at 840°C (wet-oxidation method)(Wang and Anderson, 1998).

Soil organic matter was obtained by multiplying the value for organic carbon by the "Van Bermenalen factor" of 1.724. For exchangeable bases, Ca^{2+} and Mg^{2+} was obtained by ammonium acetate (NH4 OAC) method, and Na^{+} and K^{+} by Flame photometer for cation exchange capacity (CEC) was obtained using Blakemore et al. (1987) method. Available phosphorus was obtained using Bray 11 bicarbonate extraction method as described by Olsen and Sommers (1982). Trace elements and lead were determined using Atomic Absorption Spectrophotomettry (AAS). Poly aromatic hydrocarbons were determined.

Computation

Hazard quotient (HQ): HQ expresses the possibility of the contaminant being an ecological risk or a contaminant of potential ecological concern. The hazard quotient (HQ) was calculated as follows:

HQ = Measured concentration/Toxicity reference value (Lindsay and Novell, 1977)
When HQ > 1: Harmful effects are likely due to contaminant in question
When HQ = 1: Contaminant alone is not likely to cause ecological risk
When HQ < 1: Harmful effects are not likely

Bioaccumulation quotient (BQ): This expresses the possibility of contaminant being significantly accumulated in plant parts, thereby posing health threats. It was expressed by the following equation:

BQ = Concentration of accumulated pollutant in the accumulant / Concentration of accumulated pollutant.

Plant height, leaf surface and dry matter weight of maize plant were measured.

Data analysis

Data on maize yield variables, soil physical and chemical properties were subjected to analysis of variance (ANOVA) using GenStat Discovery Edition 3, while the significant variations in the means were determined using Fischer's LSD (least significance difference) at 5% probability.

RESULTS AND DISCUSSION

Effects of contamination, location and soil depth on soil physicochemical properties

Some physical properties of the soil used for the study are presented in Table 1. Soil texture varied from sandy loam to loamy sand attributable to nature of parent materials and high rainfall that could favor washing away and leaching of silt-sized and clay-sized fractions. Similar observation has been made (Ekundayo and Obuekwe, 1997).

Soil bulk density (Bd) significantly ($P<0.05$) increased by 25%, while Ksat decreased (81%) due to effects of pollution. Increased Bd suggests compaction due to rainfall that clog the pore spaces in soils, while surface sealing arising from the presence of oil deposit on the top soil layer (hydrophobic layer) prevents water penetration. Lower Bd obtained in the uncontaminated soils is a positive productivity indicator as it helps in easing root penetration, and encourages downward movement of water through old root channels (Mbah et al., 2009). Low soil Bd and higher saturated hydraulic conductivity (Ks) as obtained in control soil could lower run-off and erosion, while increasing aeration and internal drainage (Remon et al., 2005).

Total porosity (TP) decreased by 3.1% in contaminated and increased (86.9%) in unpolluted soil. Value of available water capacity (AWC) was lower (30.6%) at Nsukka compared to Ngwo (37.7%) and Obollo Afor (43.8%) (Table 1). High AWC may be associated to the high value (18.1 %) of clay, which has the ability to retain water.

Soil pH reduced from 6.8 to 6.4 (5% reduction) in contaminated soil due to significant ($P < 0.05$) increase in Al^{3+} content. However, petroleum hydro-carbon mediated decrease in soil pH has been attributed to the production of organic acids by microbial metabolism (Osuji and Nwoye, 2007; Remon et al., 2005) as well as degradation of the hydrocarbons, which may have resulted in the release of acidic intermediates and final products that probably lowered pH of the mixture (Wang et al., 1999). Low pH have been associated to loss of exchangeable bases ($Ca^{2+,}$ $Mg^{2+,}$ K^{+}, Na^{+}) due to displacement reactions in the soil colloidal complex and excessive rainfall that could lead to eluviations and leaching loses (Ngobiri et

Table 1. Effects of contamination, location and soil depth on soil physical properties.

Soil sample	Texture	Clay%	Silt %	Fine sand %	Coarse sand %	Total sand %	TP%	KsCm/s	BDg/cm^3
Contamination									
Contaminated	LS	14.8	12.22	24.6	48.5	73.1	36	1.13	1.70
Uncontaminated	LS	8.7	8.83	24.8	46.7	71.5	49	5.81	1.36
LSD (p <0.05)		4.80	2.38	Ns	5.49	4.11		2.61	0.04
Location									
Nsukka	LS	10.8	6.63	23.01	59.8	82.8	43	4.45	1.50
Ngwo (9th mile)	LS	9.3	7.22	26.22	57.2	83.5	34	3.60	1.75
Obollo-afor	SL	18.1	10.22	24.96	46.7	71.7	49	2.36	1.35
LSD (P<0.05)		5.88	2.92	1.98	6.72	7.48	Ns	Ns	Ns
Soil Depth									
0-20cm	LS	11.8	6.55	27.02	54.6	81.6	nd	Nd	nd
20-40cm	LS	13.7	9.49	22.44	54.6	77.0	nd	Nd	nd
LSD (P <0.05)		Ns	2.38	1.61	ns	Ns	nd	Nd	nd

Ks, soil saturated hydraulic conductivity; BD, soil bulk density; TP, total porosity; AWC, available water capacity; LSD, least significant difference; NS, not significant; nd, not determined; Ls, loamy sand; SL, sandy loam.

al., 2007).

The results show organic carbon (OC) of contaminated soil to have increased significantly (P<0.05) by 196.3% relative to control (Table 2). This increase could be linked to high content of carbon in the oil, most of which are of fossil origins. Low contents of N, P and K in polluted soil (Table 2) confirms earlier report (Lehtomaker and Niemela, 1975) that showed low value of N, P and K reserve in petroleum hydrocarbon contaminated soil.

Exchangeable Ca, Mg and K decreased by 34.3%, 27 and 8.3%, respectively, in contaminated soil, suggesting displacement reaction in the cation exchange site by the active exchangeable Al^{3+}.

This result is contrary to earlier findings by Amadi et al. (1993) who reported increases in the cations of polluted soils. Lack of significance of CEC, Ca^{2+}, K^{+}, CEC, and H^{+} insinuates that contamination had no effect on these parameters.

Available P significantly (P<0.05) reduced by 40% in polluted soil (Table 2) and could be attributed to high hydrocarbon content in the automobile waste oil, which may have fixed available P, hence less availability (Ekundayo and Obuekwe, 1997).

Effects on trace elements and lead contents

The results (Table 3) show that there was heavy metal presence in contaminated soil relative to uncontaminated soil. The heavy metal composition of soil for Iron (Fe) ranged from 2.05 to 3.92 mg kg^{-1}, Mn (2.91-3.20 mg kg^{-1}), Zn (2.97-3.92 mg kg^{-1}), Pb (0.10-0.04 mg kg^{-1}), Cu (2.94-2.29 mg kg^{-1}), while Boron concentration was lower (2.63 mg kg^{-1}) in contaminated when compared with uncontaminated (3.02) soil. Both increases and/or decreases in contamination and at soil depth levels were significant (P<0.05). The increase in micro-nutrient or trace elements under contaminated soil is expected as oil pollution on soils makes some nutrients that are toxic to plants like Mn more available (Adeniyi and Afolabi, 2002; Mbah et al., 2009).

Interaction of contamination by location and by soil depth was significant (P<0.05) for all heavy metals except Zn. Increased concentration of heavy metals and especially lead (Pb) could be due to large admixture of the crude oil effluents with transmission and brake fluid and paint, gasoline, diesel, and other petrochemicals (Osu and Okereke, 2010).

Effect of oil contamination on soil poly aromatic hydrocarbon (PAH) contents

Decreases in soil poly aromatic hydrocarbon from original concentration were observed (Table 4). Acenaphthene decreased from 0.2461 to 0.2117 mg/l. The total PAH reduced from 5.8029 to 1.3425 mg/l. PAH reductions may have resulted from evaporation and microbial degradation in a dissolved state (Wang et al., 2000; Kastner and Mahro, 1996; Lehtomaker and Niemela, 1975).

Impact of remediation on maize yield factors (phyto assessment)

The hazard quotient of Zn (4.9), Cu (14.9) and Mn (3.2) was greater than unity (1), suggesting possibility of harmful effects (ecological risks) of trace elements and

Table 2. Effects of contamination, location and soil depth on soil chemical properties.

Soil sample	pH H_2O	pH Kcl	OC%	OM%	Total N%	Ca^{2+} (Cmol/kg)	Mg^{2+} (Cmol/kg)	K^+ (Cmol/kg)	Na^+ (Cmol/kg)	CEC (Cmol/kg)	H^+ (Cmol/kg)	Al^{3+} (Cmol/kg)	Avail P ($mg.kg^{-1}$)
Contamination													
Contaminated	6.44	5.98	6.07	10.46	0.09	2.78	0.68	0.11	0.21	184	0.95	0.53	9.4
Uncontaminated	6.78	6.12	1.41	3.53	0.11	4.23	0.93	0.12	0.16	147	0.73	0.3	14.23
LSD (P<0.05)	0.3	Ns	1.44	2.49	0.01	ns	0.17	Ns	0.03	Ns	Ns	0.19	2.34
Location													
Nsukka	6.51	6	4.02	6.92	0.1	1.75	0.88	0.09	0.16	153	0.68	0.45	7.46
Ngwo(9^{th} mile)	6.58	5.94	3.35	5.77	0.09	5.1	0.73	0.14	0.21	186	0.72	0.53	14.7
Obollo- afor	6.65	6.21	3.85	8.3	0.11	3.68	0.83	0.12	0.18	156	1.12	0.28	13.29
LSD(P<0.05)	Ns	Ns	Ns	Ns	Ns	2.32	Ns	0.03	0.04	Ns	Ns	Ns	2.87
Soil depth													
0- 20cm	6.72	6.07	3.43	7	0.11	3.85	1.13	0.12	0.19	158	0.97	0.38	11.75
20- 40cm	6.51	6.03	4.05	6.99	0.09	3.17	0.48	0.12	0.18	173	0.72	0.45	11.89
LSD (P<0.05)	Ns	Ns	Ns	Ns	0.01	1.9	0.17	ns	ns	Ns	Ns	Ns	Ns

OC, organic carbon; OM= organic matter, Total N= total nitrogen, Ca^{2+} = calcium, Mg^{2+} = magnesium K^+ = potassium, Na^+ = sodium, CEC = cation exchange capacity, H^+ = hydrogen, Al^{3+} = aluminum, Avail P = available phosphorus, Ns= not significant.

lead (Pb) to maize plants. Iron (Fe) quotient (0.87) was less than 1, indicating hazard effect that is not likely. These results are in line with the report by Lindsay and Norwell (1977), which showed critical levels (hazard quotient) of heavy metals for corn as: 0.8 ppm for Zn, 4.5 ppm for Fe, 1.0 ppm for Mn and 0.2 ppm for Cu.

Table 5 presents the effects of crude oil contamination on the germination and yield indicators (height and leaf area) of maize. It took longer days for maize seedling emergence on untreated polluted soils, given the value at 8 days compared to 4 days in unpolluted soils treated with compost manure (CM) (control). This suggests inhibited germination rate with higher oil pollution. At one week after sowing (1 WAS), percentage emergence of seedlings was 87.3% in control when compared to 15% of polluted soil.

The percentage survival rate in 2-3 weeks after sowing was 87.3% in control, 68.45% in unpolluted soil without CM, 32.41% in polluted soil with CM, and 8.50% in polluted soil without CM. The percentage significant (P<0.05) reduction in emergence and survival rates could be associated to a number of reasons: High critical levels (HQ>1) of the heavy metals that showed likelihood of harmful effects; the embryo of the seed been affected when in contact with crude oil, and high content of aromatics in the crude oil explaining the growth inhibition of seedlings. Similar reports have been made (Udo and Fayemi, 1975; Vwioko and Fashemi, 2005).

At eight days after sowing (8 DAS), maize seedlings were 4.2 cm long in untreated polluted soil, giving a 37.4% (-7.18 cm) decrease in comparison with the control. During the same period, leaf area significantly (P<0.05) decreased by 81.7% (-5.93 m^2) relative to manure treated unpolluted soil (Table 5). Maize seedlings on polluted soils began to turn yellow at 9 days and necrotic at 19 DAS, while those of control plot started yellowing at 3 WAS (21 days) but showed recovery signs thereafter.

There were significant (P<0.05) differences and reductions in the plant height and leaf area of maize seedlings in the polluted soils and those of non-polluted soils (Table 5). Poor growth performance of the maize seedlings in polluted soils could be due to heavy metals bioaccumulation, although not significant (Table 3). These results are in line with previous reports (Vwioko and Fashemi, 2005) that crude engine oil waste polluted soils could become unsuitable for plant growth due to a reduction in the level of available plant nutrients or rise in the toxic level of elements, all of which affected plant height and leaf area. Zinc element may be highly implicated in this study as toxicity symptoms of zinc include chlorosis and depressed plant growth.

Dry matter weight of the maize seedlings significantly (P<0.05) decreased from 6.79 g in treated unpolluted soil through 0.48 g in polluted without CM soil, representing 92.9% (-6.31 g) decrease. Better performance of the maize seedlings in unpolluted soils could be associated to added soil amendment, and which may have contributed to increased microbial activities.

Examination of the prevalent microorganisms revealed high presence of bacteria such as *Achromobacter, Clostridium, Bacillus subtilis, Sarcina and Micrococcus*; Fungi like dark green dusty colonies of *Aspergillus fumigatus, A. niger, Penicillium* spp*., Geotrichum* spp*.,* and T*richoderma* spp. as well as Actinomycetes , *Norcadia spp and Streptomycetes.* These microbes identified as active members of bioremediation microbial consortia (Ekundayo and Obuekwe, 1997; Osu and Okereke, 2010) may have been involved in the bioremediation process in this study.

Table 3. Effects of contamination, location and soil depth on soil trace elements and heavy metal.

Soil sample	Boron (ppm)	Copper (ppm)	Lead (ppm)	Zinc (meq/100g)	Manganese (ppm)	Iron (ppm)
Contamination						
Contaminated	2.63	2.98	0.10	3.92	3.20	3.90
Uncontaminated	3.02	2.29	0.04	2.97	2.91	2.05
LSD(P<0.05)	0.004	0.002	0.002	0.04	0.06	0.04
Location						
Nsukka	2.56	4.31	0.07	2.60	2.70	5.46
Ngwo(9^{th} mile)	3.17	2.13	0.10	4.10	2.35	1.68
Obollo- afor	2.75	1.46	0.04	4.20	4.20	1.82
LSD(P<0.05)	0.004	0.002	0.002	0.44	0.07	0.044
Soil depth						
0- 20cm	3.04	3.36	0.09	3.67	2.90	3.92
20- 40cm	2.61	1.91	0.05	3.60	3.27	2.05
LSD(P<0.05)	0.004	0.002	0.002	Ns	0.06	0.04

Figures in parenthesis represent heavy metal values at 4 weeks after sowing.

Table 4. The effect of soil amendment on polyaromatic hydrocarbon (PAH) content of soil.

PAH (mg/l)	Contamination (initial)	3 month
Acenaphthene	0.2461	0.2117
Cenaphthylene	0	0.2148
Anthracene	0	0
Benzo (a)pyrene	1.8062	0.5931
Chrysene	0	0
Fluorene	0	0
Naphthalene	0.1274	0
Phenanthrene	1.2082	0.3229
Pyrene	2.4150	0
Total	5.8029	1.3425

Table 5. Effect of contamination on the germination, survival rate, plant height, leaf area and dry matter yield of maize seedlings

Treatment	Number of days taken for seedling emergence	Percentage emergence at 1 WAS (%)	Height of emergent in 8; 21 DAS (cm)	Leave area (m^3) in 8; 21 DAS (cm	Percentage survival of emergent at 2WAS	1^{st} Day of noticed yellowing in plants	Dry matter weight (g)
Contamination without CM	6	14.68	4.2; 7.0	1.33; 5.6	8.51	9	0.48
Contaminated with CM	5	54.30	6.7; 11.9	2.83; 5.57	32.44	11	1.72
Uncontaminated without CM	4	72.60	11.0; 15.7	5.90; 20.14	68.45	17	4.85
Uncontaminated with CM	3	87.28	13.5; 19.2	7.26; 27.20	87.26	21	6.79
FLSD (P<0.05)			1.726; 3.467	0.947; 3.319			3.63

DAS, days after sowing; WAS, week after sowing; CM, compost manure.

Microorganisms grow on substrates such as compost manure and produce enzymes that metabolize the hydrocarbons in the compost matrix (Diaz et al., 1996). Remon et al. (2005) observed that increased activities of microorganisms cause improved enhance-ment of structure, water retention, permeability, drainage and aeration of soils.

Conclusion

This study reveals high levels of heavy metals that affected seedlings emergence, resulting to low survival percentages and high bioaccumulation quotient. There were high decreases in soil total poly aromatic hydrocarbon from original concentration. Values of maize growth parameters (height, leave surface) improved in both unpolluted and polluted soil added compost manure. Study identified active members of bioremediation microbial consortia that increased microbial activities, enhanced hydrocarbon decomposition and improved physical, chemical characteristics productivity of the soils in terms of yield.

High hazard quotients (HQ > 1) of trace elements pose ecological risks to maize production. Therefore, paradigm shift to sustainable biological soil systems management is desired. Also, mechanic villages should be situated several kilometers away from major land uses to protect soil resources, ensure environmental sanity and sustainability.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Biodegradation of hydrocarbon compounds in Agbabu natural bitumen

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The recovery of an environment polluted by petroleum and allied hydrocarbons through bioremediation is being embraced globally as the best technology of removing hydrocarbon pollutants from environment. Infrared spectral changes and gravimetric analysis from the preliminary biodegradability study carried out on Agbabu Natural Bitumen showed the vulnerability of the bitumen to some bacteria: *Pseudomonas putrefaciens*, *Pseudomonas nigrificans, Bacillus licheniformis, Pseudomonas fragi* and *Achromobacter aerogenes*. This study investigates the ability of *P. putrefaciens*, *P. nigrificans, B. licheniformis, P. fragi* and *A. aerogenes* to degrade the aliphatic and polycyclic aromatic hydrocarbon fractions of Agbabu natural bitumen. Samples of the bitumen were separately inoculated with each of the bacteria for 14 days and the hydrocarbon profiles before and after inoculation were quantified using gas chromatography technique. The total aliphatic hydrocarbon compounds (C_{11} - C_{29}) in the bitumen degraded by *P. putrefaciens* and *P. nigrificans* was slightly higher than that in the undegraded bitumen, while the concentration of compounds (C_{11} - C_{29}) found in samples of the bitumen degraded by *B. licheniformis, P. fragi* and *A. aerogenes* was less than what was contained in the undegraded bitumen. Also the even-odd carbon-ratios of the degraded bitumen were higher than unity while these were less than unity in the undegraded bitumen. The polycyclic aromatic hydrocarbons (PAHs) profile in the bitumen degraded samples also differed from that of undegraded bitumen. A substantial reduction in the concentration of some PAHs was found in the bitumen samples following their degradation by the bacteria strains, typically from 55.98 to 30.79%, thus suggesting the possibility of using the bacteria strains for bioremediation process.

Key words: Agbabu, bitumen, bacteria, biodegradability, hydrocarbons.

INTRODUCTION

Petroleum hydrocarbon compounds are the most widespread pollutants in water and soil. Some PAHs have been implicated for having the capacity to damage genetic materials and consequently leads to development of different type of cancers. High molecular weight PAHs such as benzo(a)pyrene and dibenzo(a,h)anthracene

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are the most culprits of cancer inducers (Schneider et al., 2000). Thus, the global attention is focused on how to reduce the concentrations of these in the environments. Although, there is variety of techniques to achieving this, biodegradation appears to be a promising method of recovering petroleum polluted environment. Biodegradation refers to reduction in complexity of chemical compounds with aid of biological catalysts produced by microorganisms (Wilson and Jones, 1992). Biodegradation has some advantages such as complete removal of the pollutants at low cost from the petroleum environment compared to other methods of remediation which are based on physical or chemical principle (April et al., 2000).

Many components of petroleum hydrocarbons have been reported to be biodegradable (Jain et al., 2011, Margesin and Schneider 1999b, Braddock et al., 1997). As far back as early 70s, a wide range of microbial species have been identified to have capacity to degrade hydrocarbons. Among these are soil moulds, such as *P. glaucum,* some yeast (for example *Candida utilis*) and some species of bacterium (for example, *Pseudomonas norcardia and Mycobacteria*) (Wang et al., 1998).

In biodegradation of crude oil, bacteria are the major microbes involved. The popular view on biodegradation of crude oil is that only aerobic bacteria are involved, but recent studies have shown that anaerobic bacteria too can degrade crude oil (Higgins and Burns, 1975).

When petroleum is attacked and degraded by microbes, certain fundamental changes in the composition and properties of the crude oil resulted. Among these changes are sequential and systematic removal of various hydrocarbons and other compounds, selective degradation of specific isomers within individual compound classes and the production of acidic compounds (Reneter, 1994).

It has been established that biodegradation leads to oxidation of some components of crude oil. Six carbon atoms and above are oxidized; leading to a decrease in aromatic hydrocarbon content of the oil. (Widdel and Rabus, 2001, Ian et al., 2003, Volkmen et al., 1984). Generally, in biodegradation of crude oil, the hydrocarbons are preferentially destroyed in decreasing order of: n-alkanes>branched alkanes>aromatic hydrocarbon>alicyclic hydrocarbon (Wang et al., 1998; Meredith et al., 2000).

This is followed by sulpur-oxygen- and nitrogen-containing compounds (Wenger et al., 2001; Miller et al., 1987). Products of crude oil biodegradation include acyclic, and cyclic, saturated and aromatic carboxylic acids and phenols (Ian et al., 2003; Fedorak and Lake 1984; Huang et al., 2003). A complex variety of acidic non-hydrocarbons are also produced from biode-graded aromatic heterocyclics found in oil (Taylor et al., 1990; Mackenize et al., 1983; Thorn and Alken, 1998).

Nigeria has a large deposit of natural bitumen, which is ranked to be one of the five largest deposits of natural bitumen in the World (Adegoke, 2000).The bitumen deposit is located in the bitumen belt which spans across three states in Nigeria, but very often it is referred to as Agbabu Natural Bitumen (ANB), being the town (Agbabu) where the Nigerian natural bitumen was first discovered in 1900 (Adedimila, 2000). The full exploitation of this important engineering material has not commenced, however, intensive scientific research investigations on the material are currently being carried out by experts with a view to providing useful information for prospective investors.

Infrared data and gravimetric analysis from our previous study showed that, *P. putrefaciens*, *P. nigrificans, B. licheniformis, P. fragi* and *A. aerogenes* impacted qualitative and quantitative changes on ANB (Olabemiwo et al., 2011). The present study investigated the ability of these bacteria to degrade the aliphatic and polycyclic aromatic hydrocarbons. The objective is to provide information that would serve as template for the development of appropriate bioremediation scheme for the Agbabu bitumen spills in particular and petroleum hydrocarbons in general

MATERIALS AND METHOD

Biodegradation

The biodegradation experiment was as described in our earlier study (Olabemiwo et al., 2011). Summarily, this involved the isolation, purification and characterization of the bitumen degrading bacteria strains. (*P. putrefaciens*, *P. nigrificans, B. licheniformis, P. fragi* and *A. aerogenes*). Sample of bitumen was then inoculated with each of the bacteria strains for 14 d. The biodegraded/residual bitumen was subsequently harvested and kept at 4°C, for further analysis.

Fractionation of the biodegraded bitumen

Samples of fresh and degraded bitumen isolated from biodegradation experiment were separately dissolved in dichloromethane (DCM). The resulting solution was then fractionated into aliphatic, aromatic and polar fractions. The fractionation was achieved through column chromatography. Activated Silica gel (70-230 mesh) was packed into 100 mL chromatographic column. Added to the top of the silica in the column was 1 g of anhydrous sodium sulfate (Na_2SO_4). This was followed by the conditioning of the system using 20 ml hexane. Thereafter, the concentrated solution of dissolved degraded bitumen was introduced into the column. The sample was eluted with 3×25 ml of *n*-hexane followed by 3×25 ml DCM: *n*-hexane (95:5) to obtain the aliphatic and aromatic fractions, respectively. Eluates from each fraction were pooled together and rotary evaporated and kept in labeled sample vials at 4°C till analysis.

Gas chromatography

Analysis of aliphatic and aromatic fractions of residual oil were separately carried out on a 5890 series II Hewlett Packard gas chromatograph equipped with flame ionization detector (FID). A fused silica capillary column (30 m × 0.25 µm) coated with 0.25 µm

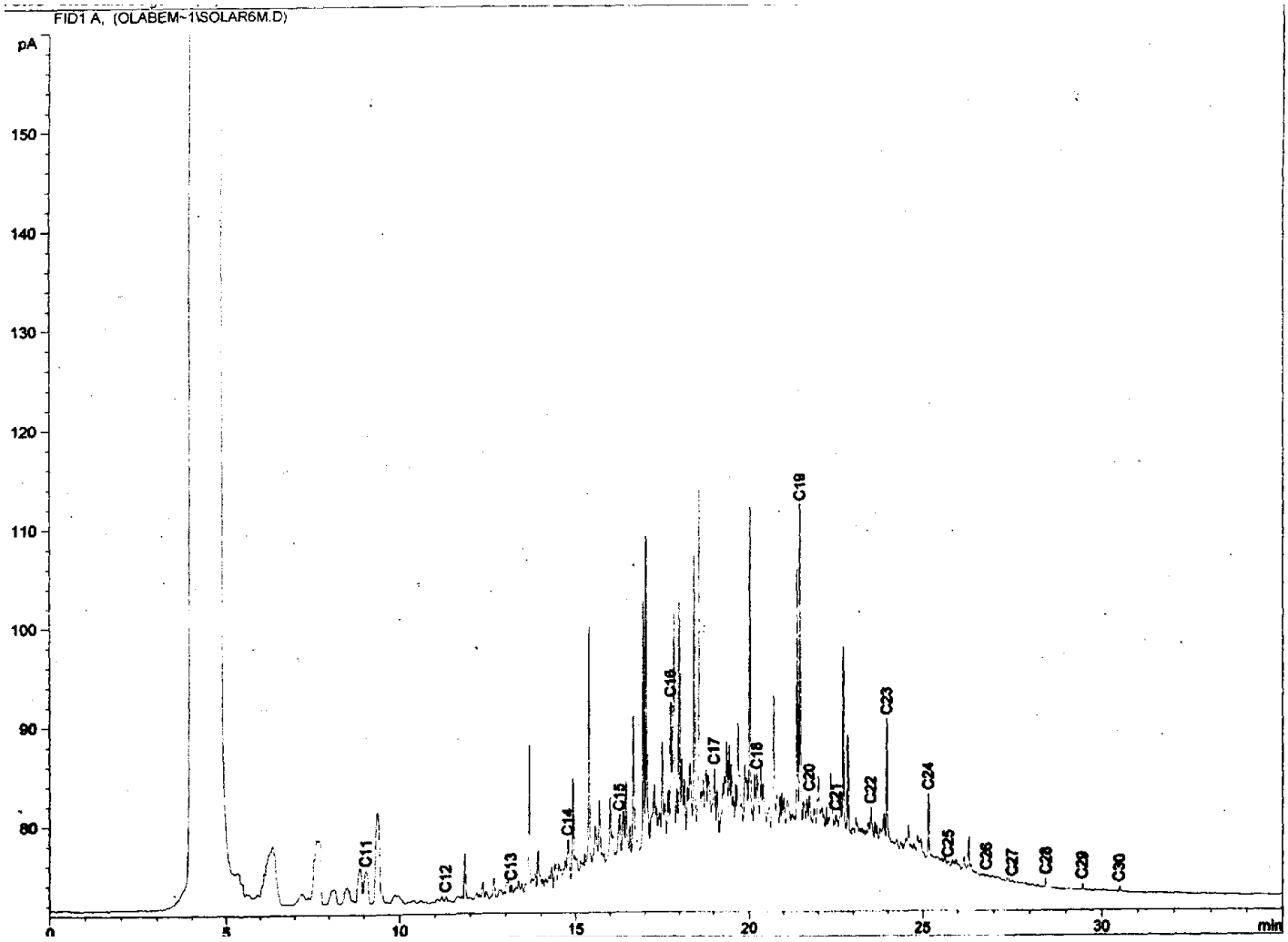

Figure 1. A typical chromatogram of degraded sample of ANB.

film of HP-5 was used. The chromatograph was powered with HPCHEM software. The instrumentation for quantification of aliphatic hydrocarbons was as follows: Nitrogen was used as carrier gas with a pressure of 30 psi. The column temperature was programmed with initial temperature of 60°C, held isothermally for 2 min and then increased to 260°C at the heating rate of 10°C/min for 20 min. It was held at this temperature for 2 min, thereafter, increased to 320°C at heating rate of 12°C/min for 5 min and held at this temperature for 2 min. The injector and detector temperatures were maintained at 250°C and 350°C, respectively. For the aromatic components, the column temperature started at 68°C and was held at this temperature for 2 min, thereafter, the temperature increased to 260°C at heating rate of 12°C/min for 16 min. It was held isothermally at 260°C for 4 min and thereafter increased to 320°C at heating rate of 15°C/min for 4 min and held at 320°C for 8 min. The carrier gas was nitrogen at a pressure of 35 psi. Hydrogen and air were supplied at 25 and 30 psi, respectively. Injector and detector temperatures were 300 and 320°C respectively and the volume of sample injected was 2 µL. Calibration curves for the aliphatic and polycyclic aromatic hydrocarbons were prepared using their standard solutions which were supplied by the manufacturer of the equipment.

RESULTS AND DISSCUSSION

A typical chromatogram of aliphatic hydrocarbons of ANB sample degraded by one of the bacteria used in this study is presented in Figure 1. The total and the distribution of individual aliphatic hydrocarbons (C_{11} - C_{29}) in ANB degraded by *P. putrefaciens, P. nigrificans, B. licheniformis, P. fragi* and *A. aerogenes* are presented in Table 1 and Figure 2. It can be observed that the total and distribution of individual aliphatic hydrocarbons vary with the type of bacteria used. The total and distribution of individual aliphatic hydrocarbons also differed from what was contained in the control sample (undegraded ANB). This is a very good evidence of vulnerability of the ANB to biodegradation and is in agreement with our previous findings (Olabemiwo et al., 2011). The distribution of individual aliphatic hydrocarbons (C_{11} - C_{29}), found in the degraded ANB is presented in Figure 2.

The biodegradative activity of *P. putrefaciens* and *P.*

Table 1. Proportion of odd and even numbered carbon atoms in ANB

Parameter	O	A	B	C	D	E
TOCA (%)	37.44	83. 28	82.97	75.78	80.40	79.70
TECA (%)	62.56	16.72	17.03	24.22	19.60	20.30
TALPH (g)	484.73	520.95	502.99	421.53	378.48	334.29
ROENA: TOCA/TECA	0.60	4.98	4.87	3.13	4.10	3.73

TOCA, Total Odd Numbered Carbon Atoms; TECA, Total Even Numbered Carbon Atoms; ALPH, Total Aliphatic Hydrocarbons; ROENA, Ratio of total Odd Numbered carbon Atoms to Total even numbered Carbon Atom; O, controls; A, ANB sample degraded by *Pseudomonas putrefaciens*; B, ANB sample degraded by *Pseudomonas nigrificans;* C, ANB sample degraded by *Bacillus licheniformis*; D, ANB sample degraded by *Pseudomonas fragi*; E, ANBB sample degraded by *Achromobacter aerogenes.*

nigrificans caused a relatively small increase in the total aliphatic hydrocarbons (TALPH) in the degraded ANB compared to the undegraded ANB (Table 1). The two bacteria probably converted higher aliphatic hydrocarbons (> C_{30}) to lower ones as evident by the increase in the abundance of some low molecular mass aliphatic hydrocarbons (C_{11} and C_{12}) (Figure 2). The degradative activity of the *B. licheniformis, P. fragi* and *A. aerogenes* caused a decrease in TALPH found in the ANB (Table 1). The lower molecular weight aliphatic hydrocarbons present were probably oxidized to carbon (IV) oxide and water (Obire and Nwaubeta, 2001) by these bacteria.

In the undegraded ANB, the even-odd numbered C-atoms was less than unity, while it was found to be greater than unity in all the biodegraded samples of ANB (Table 1). This implies that all the bacteria used in this work attacked mainly, the even-numbered carbon atoms in the ANB. The percentage of even-numbered carbon atoms are 17, 17, 24 and 20 in ANB samples degraded by *P. putrefaciens*, *P. nigrificans*, *B. licheniformis*, *P. fragi* and *A.* aerogenses, respectively, whereas it was about 63% in control sample. The degradative activity of all the bacteria used in this study, on the ANB led to the production of large quantity of lower molecular mass odd-numbered aliphatic hydrocarbons. This might account for the reversal of the proportion of odd to even carbon atom in the degraded samples (Figure 2). About ten-folds of aliphatic hydrocarbon with twenty-nine carbon atoms found in the undegraded ANB was found in ANB samples degraded by *P. putrefaciens* and *P. licheniformis*. Eight and five folds of this aliphatic hydrocarbon (C_{29}) were detected in ANB samples degraded by *B. licheniformis* and *P. fragi* respectively (Figure 2). On the other hand, the aliphatic hydrocarbon (C_{29}) was not detected in sample of ANB degraded by *A. aerogenes*.

The total PAHs and distribution of individual PAH in control and degraded samples of ANB are given in Figure 3. The total PAHs in each of the biodegraded samples was less than the total PAHs in the undegraded sample of ANB. Samples of ANB degraded by *P. putrefaciens* and *P. nigrificans* contained almost half the total PAHs in the undegraded ANB. In case of samples of ANB degraded by *B. licheniformis*, *P. fragi* and *A. aerogenes*, one third of the total PAHs contained in undegraded PFB was found in them. This implies that the three bacteria were able to degrade more than 60% of the total PAHs contained in undegraded ANB. The extent of degradation of PAHs achieved in this work is very close to the results of Gokcen et al. (2008), where degradation rates in the range of 42-59% were reported for some PAHs.

The distribution pattern of individual PAH in the biodegraded ANB samples also differed from that in the undegraded ANB (Figure 4). Naphthalene was found in undegraded ANB and samples of ANB degraded by *P. fragi* and *A. aerogenes*. Samples of ANB degraded by *P. putrefaciens*, *P. nigrificans* and *B. licheniformis* had no detectable quantity of naphthalene.

There were 2-, 3- and 4- fused ring PAHs in the ANB (Figure 4), prior to the degradation experiment. However, in the ANB samples degraded by *P. putrefaciens*, *P. nigrificans* and *B. licheniformis*, only 3- and 4- fused rings PAHs were detected. The ANB samples degraded by *P. fragi* and *A. aerogenes* had 2-, 3-, 4- and 5- fused rings PAHs. The 5- fused rings PAHs found in degraded samples may be due to polymerization or recombination of 2-, and 3- fused rings PAHs (Stern and Stern, 1971; Okerentugba and Ezeronye, 2003).

It can therefore be said that, all the bacteria used in this work displayed the capability of degrading some of the PAHs found in ANB. However, *B. licheniformis*, *P. fragi* and *A. aerogenes* appeared to be more efficient in degrading the PAHs in the ANB than the other two bacteria. As reported above for aliphatic hydrocarbons, the bacteria used in this work had also affected the total and distribution of individual PAHs detected in the ANB. These findings quite agree with our earlier preliminary study on biodegradation of ANB using infrared spectroscopy (Olabemiwo et al., 2011). The changes in the infrared spectrum and chromatographic analysis of the degraded ANB corroborate each other to show that the composition and structure of the ANB were altered by the bacteria used in this work.

The biodegradability of petroleum hydrocarbons by some bacteria strains have also been established by some other workers (Bushnell and Haas, 1941, Pahthapingam et al., 1998, Obire, 1990). The findings of

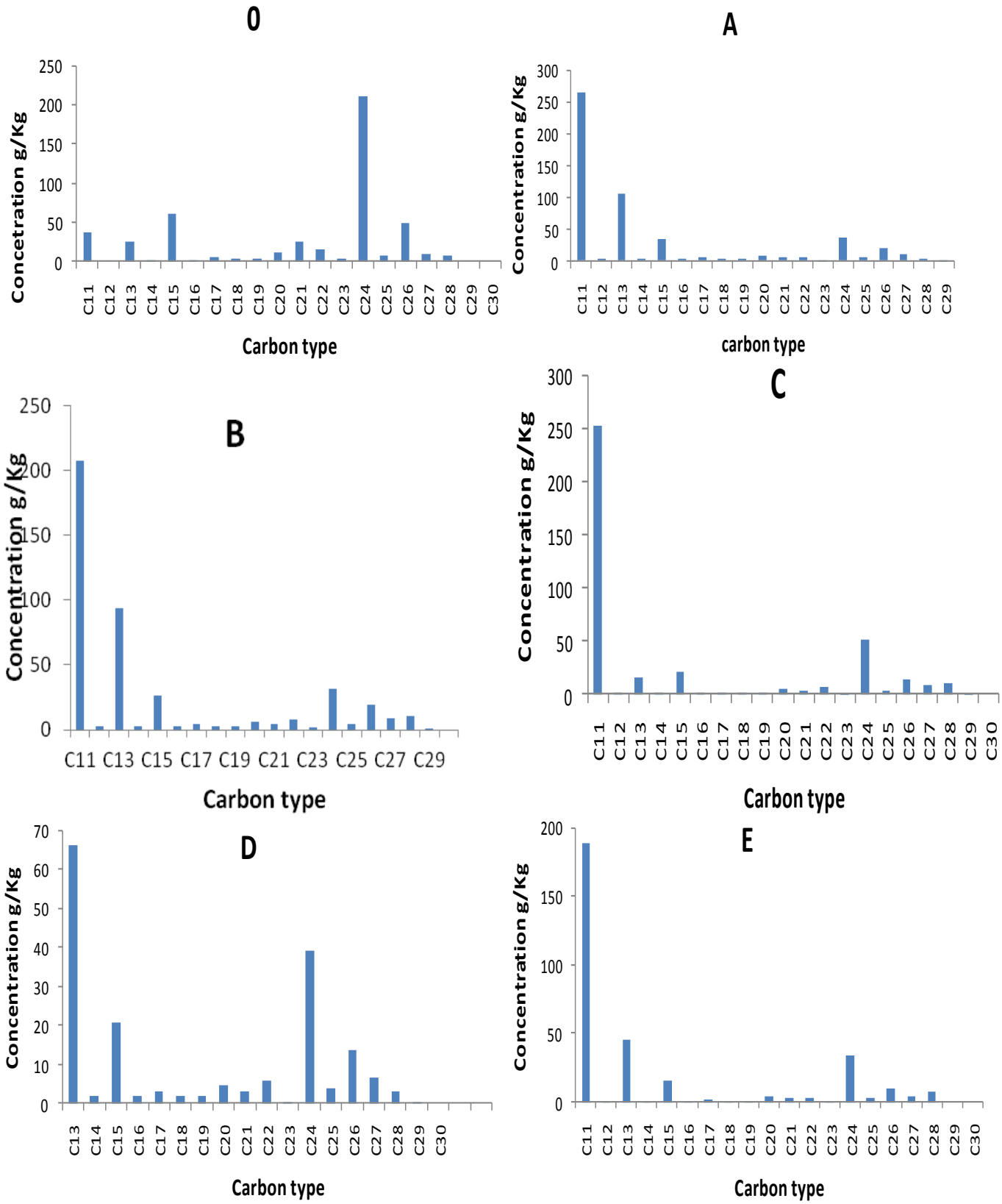

Figure 2. Distribution of aliphatic hydrocarbons in ANB degraded by some Bacteria. O = controls,A = PFB sample degraded by *Pseudomonas putrefaciens* B = PFB sample degraded by *Pseudomonas nigrificans* C = PFB sample degraded by *Bacillus licheniformis* D = PFB sample degraded by *Pseudomonas fragi* E = PFB sample degraded by *Achromobacter aerogenes.*

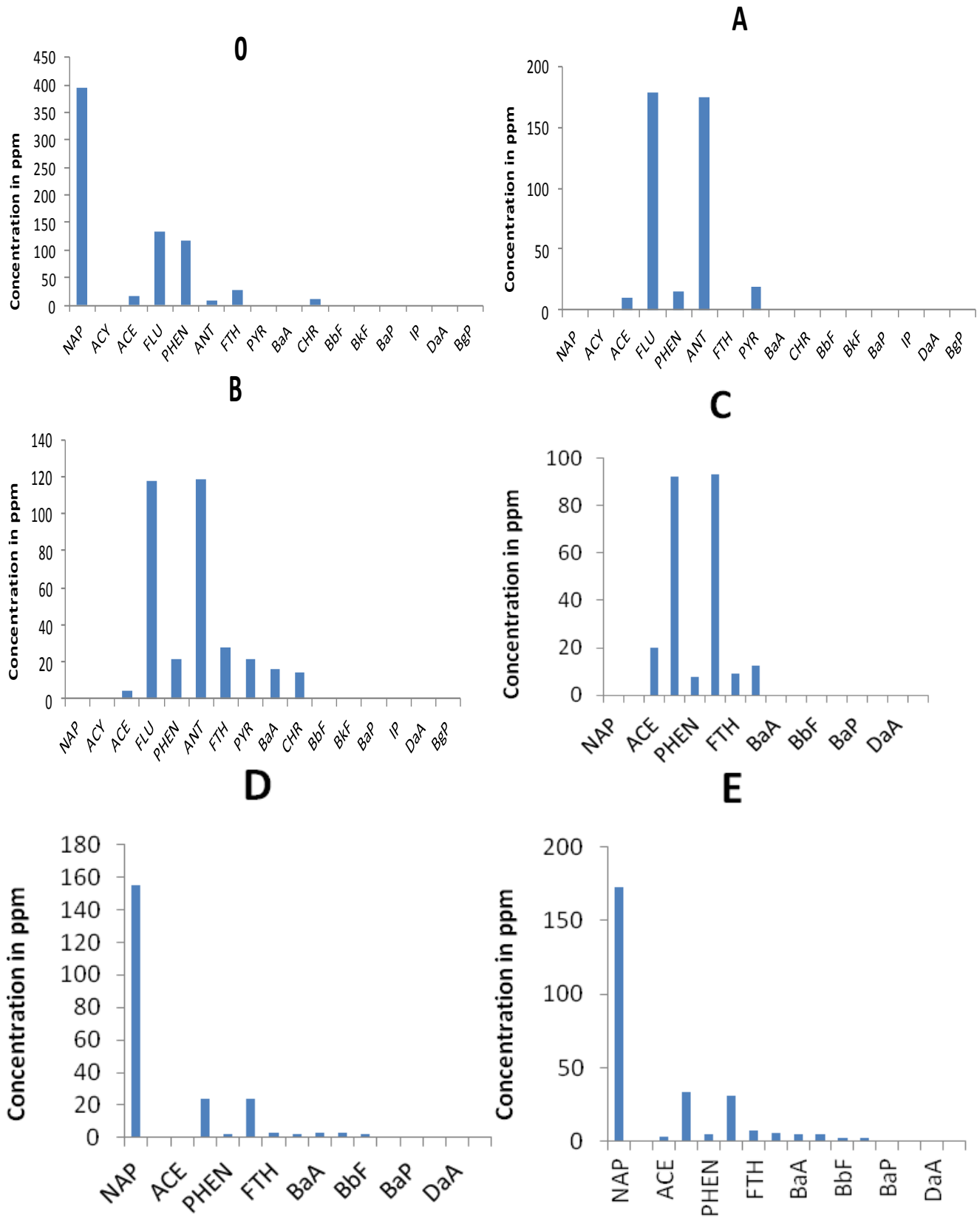

Figure 3. Polycyclic Aromatic Hydrocarbon (PAH) Profile of Biodegraded PFB. O = control,A = ANB sample degraded by *Pseudomonas putrefaciens*,B = ANB sample degraded by *Pseudomonas nigrificans*,C = ANB sample degraded by *Bacillus licheniformis*,D = ANB sample degraded by *Pseudomonas fragi*,E = ANB sample degraded by *Achromobacter aerogenes*

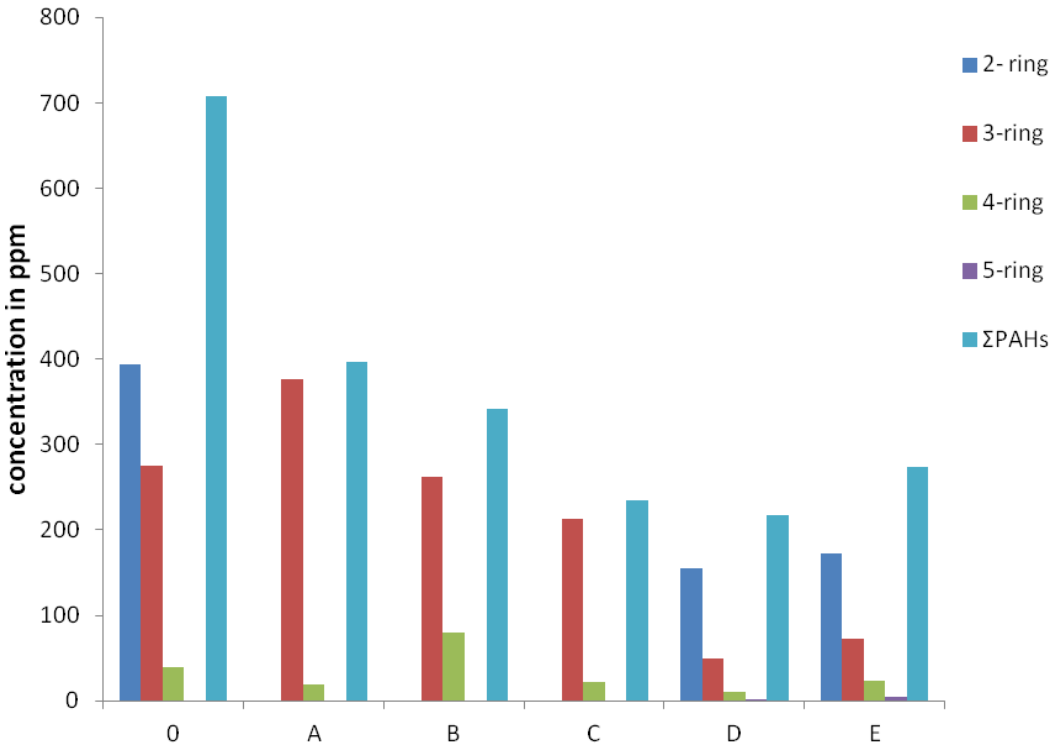

Figure 4. Group distribution of PAHs in biodegraded PFB. O = control,A = ANB sample degraded by *Pseudomonas putrefaciens*,B = ANB sample degraded by *Pseudomonas nigrificans*,C = ANB sample degraded by *Bacillus licheniformis*,D = ANB sample degraded by *Pseudomonas fragi*,E = ANB sample degraded by *Achromobacter aerogenes*.

this study show that *B. licheniformis*, *P. fragi* and *A. aerogenes* had potential of being useful in bioremediation of hydrocarbons in bitumen spill.

Conclusion

All the five bacteria strains used in this study altered the hydrocarbon profiles in ANB. However, three of these bacteria strains (*B. licheniformis*, *P. fragi* and *A. aerogenes)* reduced the total aliphatic and polycyclic aromatic hydrocarbons contents of the bitumen. Thus, these bacteria strains can be employed for bioremediation of bitumen spill in Agbabu area and its environs.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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In vivo study of lens regeneration in *Rana cyanophlyctis* under influence of vitamin A and ascorbic acid

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After removal of lens from eye of *Rana cyanophlyctis*, the pigmented epithelial cells of dorsal iris started to proliferate and dedifferentiate and consequently regenerate lens. Vitamin A and ascorbic acid enhanced the percentage lens regeneration not only in young tadpoles but also in froglets. Lens regeneration ability declined with age of animals in both control as well as treated groups.

Key words: *Rana cyanophlyctis,* pigmented epithelial cells, vitamin A, ascorbic acid.

INTRODUCTION

Urodele amphibians are unique among vertebrates in their strong ability to regenerate lost part/ parts of body throughout their lives (Ferretti and Geraudie 1998; Wolpert et al., 1998) and in their striking resistance to tumor occurrence (Okamoto, 1987, 1988, 1997; Brockes, 1998). After lens removals through the pupil of the newt's eye, pigmented dorsal iris epithelial cells begin to dedifferentiate, proliferate and transdifferentiate into lens cells (Wolff, 1895; Kodama and Eguchi, 1995). In this Wolffian lens regeneration, it is noteworthy that only the dorsal iris can regenerate a new lens, while the ventral iris never shows such ability. Despite many morphological and biochemical studies on the process of lens regeneration, the difference between dorsal and ventral cells remains to be elucidated (Reyer, 1977; Yamada, 1977). In the present study, we proved that pigmented epithelial cells of dorsal iris have intrinsic ability to transdifferentiate into lens. Vitamin A and ascorbic acid was found to accelerate the percentage of lens formation from the dorsal iris in *in vivo* condition.

MATERIALS AND METHODS

For the present experiment, three different developmental stages: young tadpoles (3 toe stage), mature tadpoles (5 toe stage) and froglets of the frog, *Rana cyanophlyctis* were employed. For study of lens regeneration in young and mature tadpoles and froglets of the *R. cyanophlyctis* under the influence of vitamin A and ascorbic acid, animals of each group were divided as per plan of experiment (Table 1). Each age group contained 20 animals and thus the present experiment was performed on 180 animals. Each group contained controls (animals were not treated with vitamin A and ascorbic acid). In control group, animals particularly froglets, were given sham injection while in treated group, animals the respective chemicals (vitamin a and ascorbic acid) were injected on alternate day after operation upto the day of termination of the experiment.

RESULTS

The results are shown in Table 2. The experiment concerned the study of lens regeneration in young tadpoles, mature tadpoles and froglets of *R. cyanophlyctis* under the influence of vitamin A and

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Abbreviations: YT, Young tadpole; **PECs,** pigmented epithelial cells; **RABP,** retinal binding proteins; **CRABP,** cellular retinoic acid binding protein; **CRBP,** cellular retinal binding protein; **NBQ,** 3-nitro benzothiazolo quinolinium chloride.

Table 1. Plan of experiment.

Group (age of animals at the time of operation)	Subgroup	Number of animals used	Day of preservation
G1-Young tadpoles (YT)	Control (YTG1C)	20	3 7 15 40
	Vitamin A treated (YTG1VA)	20	-do-
	Ascorbic acid treated (YTG1AA)	20	-do-
G2-Mature tadpoles (MT)	Control (MTG2C)	20	3 7 15 40
	Vitamin A treated (MTG2VA)	20	-do-
	Ascorbic acid treated (MTG2AA)	20	-do-
G3-Froglets (FT)	Control (FTG3C)	20	3 7 15 40
	Vitamin A treated (FTG3VA)	20	-do-
	Ascorbic acid treated (FTG3AA)	20	-do-

Table 2. Influence of vitamin A and ascorbic acid on lens regeneration in young, mature tadpoles and froglets of the frog *Rana cyanophlyctis*.

Group	Sub group	Number of animals used	Day of preservation	Number of animals preserved	Number of		Percentage (%) of regeneration
					Regenerates	Non-regenerates	
G_1-Young tadpoles (YT))	Control (YTG_1C)	20	3	4	-	4	60.00
			7	4	3	1	
			15	4	3	1	
			40	8	6	2	
	Vitamin A treated (YTG_1VA)	20	3	4	4	-	95.00
			7	4	4	-	
			15	4	4	-	
			40	8	7	1	
	Ascorbic acid treated (YTG_1AA)	20	3	4	3	1	85.00
			7	4	4	-	
			15	4	4	-	
			40	8	6	2	
G_2-Mature tadpoles (MT))	Control (MTG_2C)	20	3	4	1	3	55.00
			7	4	2	2	
			15	4	2	2	
			40	8	6	2	
	Vitamin A treated (MTG_2VA)	20	3	4	3	1	80.00
			7	4	3	1	
			15	4	4	-	
			40	8	6	2	
	Ascorbic acid treated (MTG_2AA)	20	3	4	3	1	75.00
			7	4	3	1	
			15	4	4	-	
			40	8	5	3	

Table 2. Contd.

G_3-Froglets (FT)	Control (FTG_3C)	20	3	4	-	4	20.00
			7	4	1	3	
			15	4	1	3	
			40	8	2	6	
	Vitamin A treated (FTG_3VA)	20	3	4	-	4	50.00
			7	4	1	3	
			15	4	3	1	
			40	8	6	2	
	Ascorbic acid treated (FTG_3AA)	20	3	4	1	3	45.00
			7	4	2	2	
			15	4	2	2	
			40	8	4	4	

ascorbic acid. After lentectomy, the operated animals were treated with vitamin A (30 IU/ml) and ascorbic acid (50 µg/ml) and preserved at various intervals of time (day 3, 7, 15, 40) for histological evaluation. Results were compared with that of untreated control group animals of the same age. Simultaneously, lens regenerative capability of different age group animals was also studied.

The results obtained in the present study show that lens regenerative power is present in the tadpoles of *R. cyanophlyctis.* However, it declined with the age of animal. It was 60% in young tadpoles; 55% in mature tadpoles and 20% in froglets of frog *R. cyanophlyctis.* In contrast to it, vitamin A and ascorbic acid were found to induce and accelerate lens regeneration in the animals of all ages. It was 95, 80 and 50% in young tadpoles, mature tadpoles and froglets, respectively whereas in ascorbic acid treated animals, the percentage of lens regeneration was 85, 75 and 45% in young tadpoles, mature tadpoles and froglets, respectively (Table 2). The declined trend of regeneration was also found in vitamin A and ascorbic acid treated animals with their age. The histological changes occurred in the lens regeneration of young tadpoles of sub group YTG_1C, YTG_1VA and YTG_1AA as follow: in the animals of this age group, lens regenerative events were found almost similar except their percentage. The events that occurred during lens regeneration are presented here through selective representative animals that were preserved at different time intervals. The representative cases were taken from vitamin A treated groups. In all cases (control as well as vitamin A and ascorbic acid treated animals), lens regeneration occurred from pigmented epithelium cells of dorsal iris. During lens regeneration, it was observed that after lentectomy, the two layers of pigmented epithelium of dorsal iris began to thicken and cleft arose between inner and outer lamella (Figures 1 and 2).

Figure 1 and 2 show the thickening of iris layers and cleft formation. The nuclei of iris cells change their shape on day 3 after operation. Soon the cells become elongated and the nuclei become more prominent. Later on the pupillary margin becomes knob-like. This knob-like structure continued until the free margin became swollen loop-like structure by day 7 after operation (Figure 3). Scattered mitotic figures were also observed. All these changes continue up to day 7 after operation in vitamin A treated animals. Then the cells of dorsal iris started to dedifferentiate. They throw out their melanosomes and these melanosomes later on disappeared probably ingested by macrophages. Dorsal iris cells continue to divide forming dedifferentiated cells in the region of cleft (Figure 3). These dedifferentiated cells are considered as lens forming cells. The dedifferentiated iris cells by day 15 started synthesizing the differentiated products of lens crystalline proteins. These proteins were almost similar as in normal lens development. Once the new lens had been formed, the cells of dorsal iris ceased mitosis. Figures 4 and 5 show the newly formed vesicular cellular lens attached to the margin of dorsal iris. The cells in the wall of the vesicle are cuboidal and slightly taller but in addition to the fibre growing area, was usually more than one cell deep. The central cavity varies in size due to a plug of cells that pushed as a growth from the inner layer of iris into the neck of vesicle. Now onwards by day 15 after operation, lens fibre differentiation begins. Figures 6, 7 and 8 show the cellular regenerated lens still attached to the dorsal iris. The next phase of this development was shown by differentiation of lens fibres. On day 15 onwards, primary lens fibres started to appear (Figure 9). The primary lens fibres pushed to front of the vesicle forming a nucleus behind the lens epithelium.

Now the secondary lens fibres begin to differentiate but do not enclose entirely the primary lens fibre nucleus. At last the nuclei of the secondary lens fibres progressively disappeared. It covered a long period in which the further growth of the lens carried towards final size. The final phase was the period of growth which continued about 40 days after operation. The nuclei of lens fibres eventually degenerated (Figures 10 and 11). Figure 12 shows

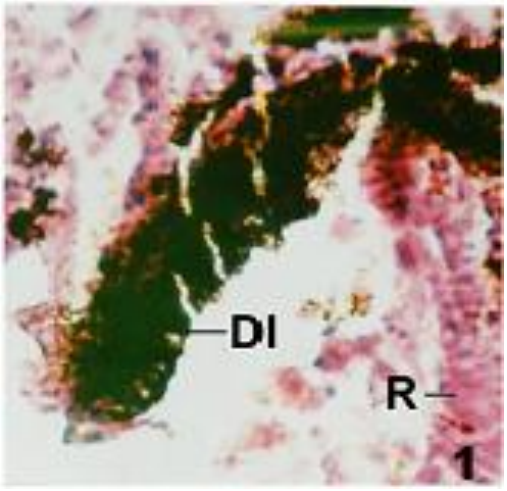

Figure 1. Microphotograph of a section passing through the lentecto-mized eye of vitamin A treated young tadpole of *Rana cyanophlyctis* showing bylaminal layers of iris (100X). DI, Dorsal iris; R, retina.

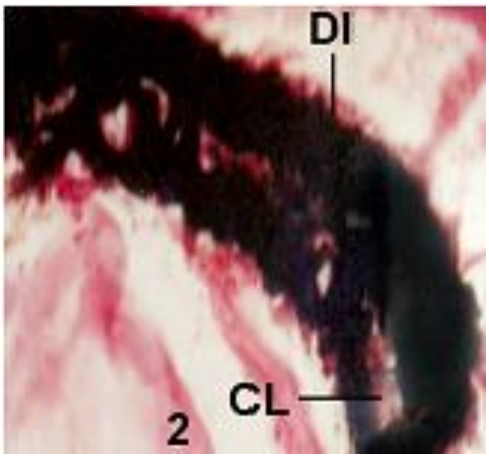

Figure 2. Microphotograph of a section passing through the lentecto-mized eye of 3 days vitamin A treated young tadpole showing cleft formation at the tip of dorsal iris (100X). DI, Dorsal Iris; CL, cleft.

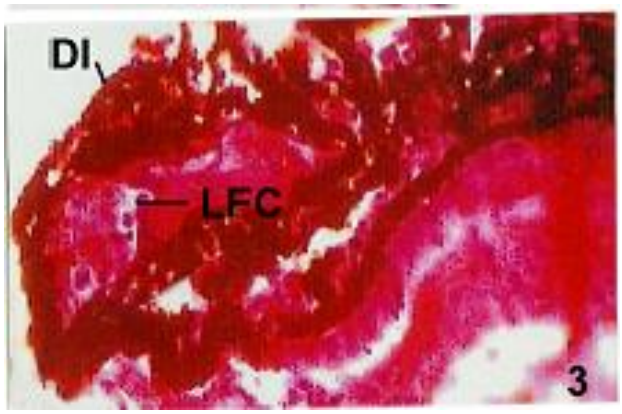

Figure 3. Microphotographs of sections passing through the lentec-tomized eye of 7 days vitamin A treated young tadpole of *Rana cyanophlyctis* showing didifferentiation of PECs and formation of lens forming cells into the cleft (100X). DI, Dorsal Iris; LFC, lens forming cells.

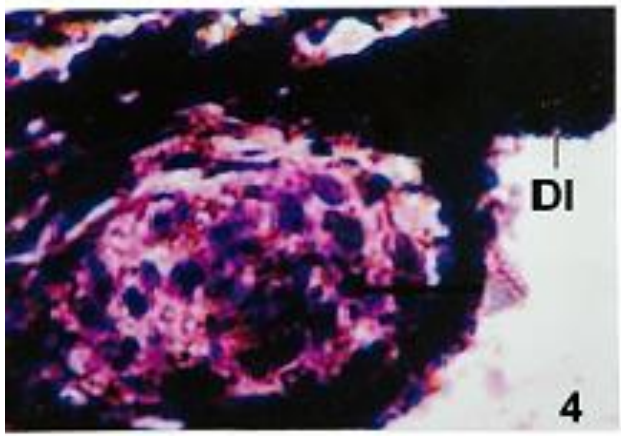

Figure 4. Microphotographs of sections passing through the lentec-tomized eye of 7 days vitamin A treated young tadpole of *Rana cyanophlyctis* showing differentiation of PECs and formation of lens forming cells into the cleft (100X). DI, Dorsal Iris.

concentrically arranged well differentiated secondary lens fibres in ascorbic acid treated animals. Similarly, well formed lens were also observed in untreated control group tadpoles (Figure 13). All these representative figures (Figures 11 to 13) clearly show well differentiated regenerated lenses in which secondary lens fibres are enclosing the primary lens fibre nucleus.

Similar types of changes have also been observed in regenerating cases of ascorbic acid treated animals and untreated control group tadpoles as well. In the control group, lens regeneration occurred from dorsal iris (Figure 13). The stages of lens fibre differentiation were found exactly similar as reported in vitamin A and ascorbic acid treated animals. However, the percentage of lens regeneration was found low in controls. Lentoids have also been observed in tadpoles of all the groups particularly of mature tadpoles and froglets (Figure 14). The dorsal iris was found unchanged in non-regenerating cases as shown in Figure 15.

Lens regeneration in the animals of G_2 group (5 toe stage mature tadpoles)

The animals of this group were of mature tadpole stage. Similar to sub group YTG_1C, YTG_1VA and YTG_1AA, the

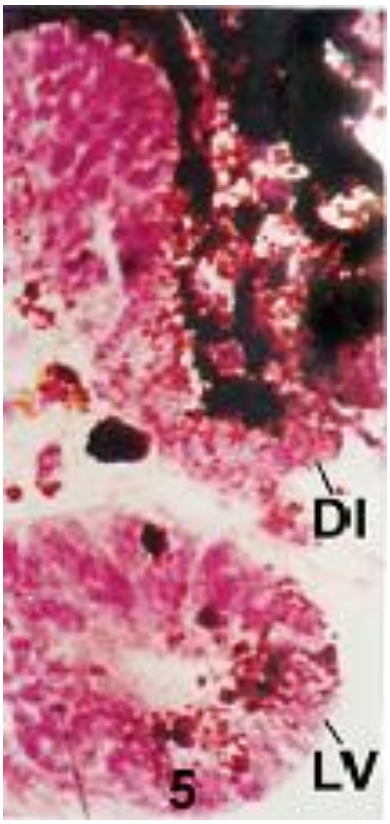

Figure 5. Microphotograph of a section passing through the lencto-mized eye of 15 days vitamin A treated young tadpole of *Rana cyanophlyctis* showing formation of lens vesicle at the tip of dorsal iris (200X). LV, lens vesicle; DI, Dorsal Iris.

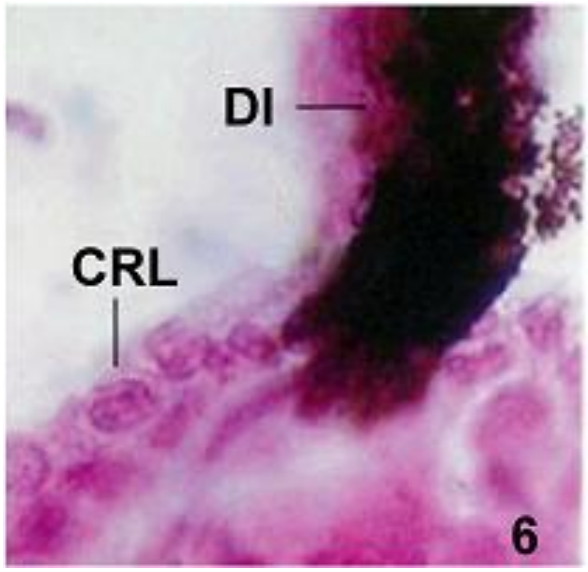

Figure 6. Microphotographs of sections passing through the lentec-tomized eye of 15 days vitamin A treated tadpole of *Rana cyanophlyctis* showing cellular regenerated lens (RL) attached with the tip of dorsal iris (200X). CRL, Cellular regenerated lens; DI, dorsal Iris.

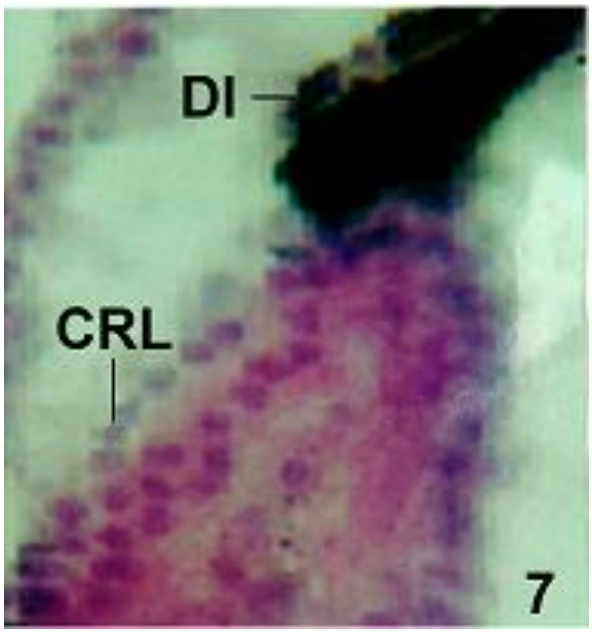

Figure 7. Microphotographs of sections passing through the lentec-tomized eye of 15 days vitamin A treated tadpole of *Rana cyanophlyctis* showing cellular regenerated lens (RL) attached with the tip of dorsal iris (200X). DI, Dorsal Iris; RL, regenerated lens.

animals of this group were subjected to lentectomy in the right eye and were divided into three sub groups MTG_2C, $MTG_2VA$and MTG_2AA. The animals of MTG_2C sub group were not treated with any chemical and were considered as control group animals. Only sham injections were given after their lentectomy whereas the animals of sub group MTG_2VA and MTG_2AA were treated with vitamin A (30 IU/ml) and ascorbic acid (50 µg/ml) after their lentectomy.

In the animals of the control sub group, MTG_2C lens regeneration occurred in 11 out of 20. However, the percentage of lens regeneration was found high (80 and 75%) in vitamin A and ascorbic acid cases. The regenerated lenses were almost similar to that of young tadpole (YT) group animals as described earlier but in few cases regenerated lenses were found smaller and were of lentoid shape. Histological events of lens regeneration were found almost similar as described earlier viz. after lentoctomy depigmentation of pigmented epithelium of dorsal iris, dedifferentiation of pigmented epithelial cells (PECs), proliferation, lens vesicle formation, differentiation of primary lens fibres and secondary lens fibres. The regenerated l enses so formed started

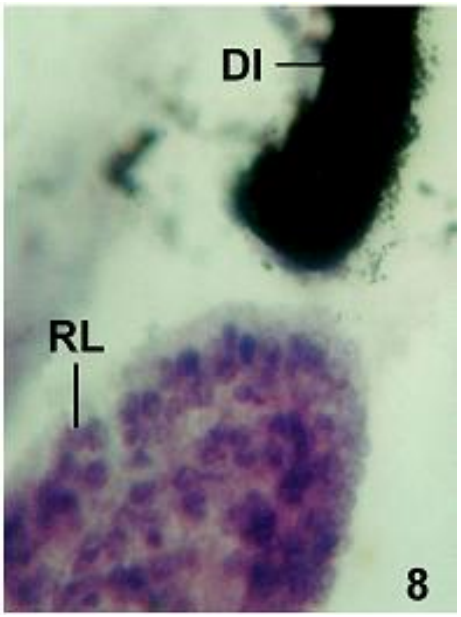

Figure 8. Microphotograph of a section passing through the lentec-tomized eye of 15 days vitamin A treated young tadpole showing further differentiation of regenerated lens (RL) (200X). DI, Dorsal Iris; RL, regenerated lens.

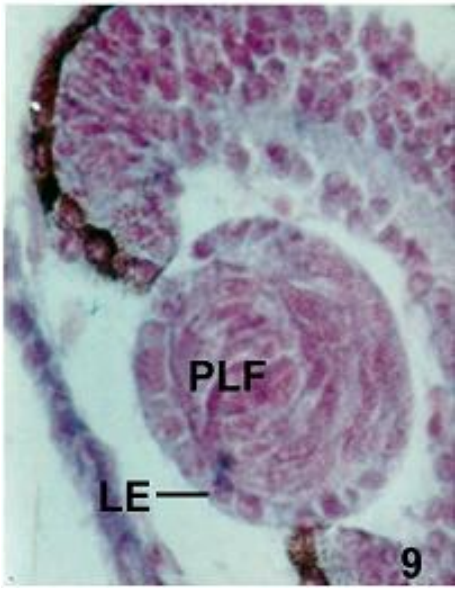

Figure 9. Microphotograph of a section passing through the lentec-tomized eye of 15 days vitamin A treated young tadpole showing regenerated lens with primary lens fiber (PLF) (200X). LE, Lens epithelium; PLF, primary lens fiber.

to grow and attend similar size and shape to normal lenses.

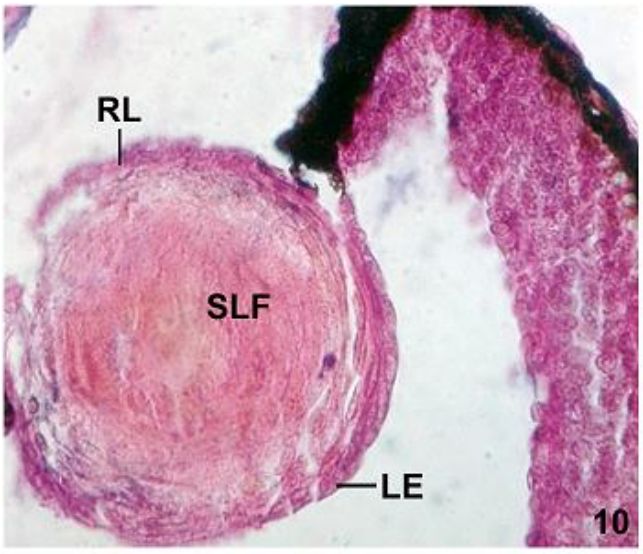

Figure 10. Microphotograph of a section passing through the lentec-tomized eye of 40 days vitamin A treated young tadpole showing regenerated lens (RL) with epithelium and secondary lens fibers (200X). LE, Lens epithelium; RL, regenerated lens; SLF, secondary lens fiber.

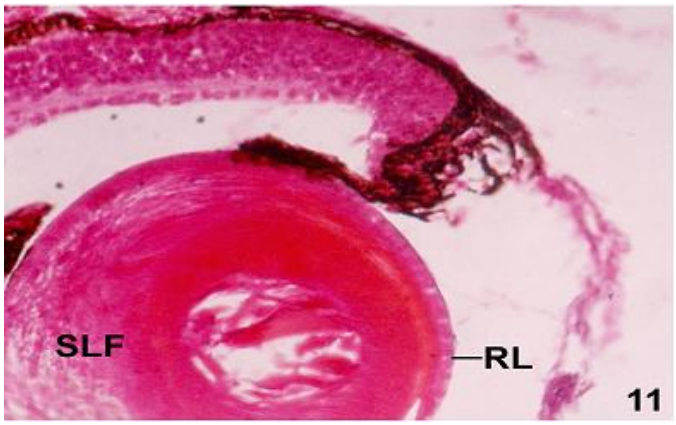

Figure 11. Microphotograph of a section passing through the lentecto-mized eye of 40 days vitamin A treated tadpole showing well differentiated regenerated lens (100X). RL, Regenerated lens; SLF, secondary lens fiber.

Lens regeneration in the animals of G_3 group (Froglet stage)

The animals employed were newly metamorphosed froglets. The method of lentectomy and mode of treatment was the same as employed for the animals of G_2 group. After lentectomy, operated animals were divided into three sub groups: FTG_3C, FTG_3VA and FTG_3AA controls, vitamin A and ascorbic acid treated animals, respectively. Normal lens regeneration was not reported in any animal of FTG_3C control group. However,

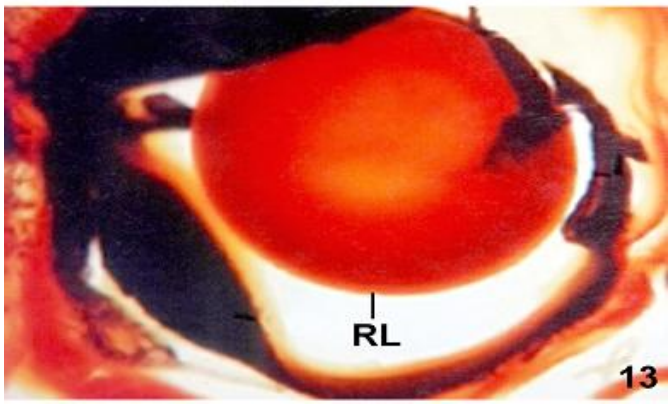

Figure 13. Photograph of a hand section of lentectomized eye of untreated tadpole (control) showing well differentiated and spherical regenerated lens (50X). RL, Regenerated lens.

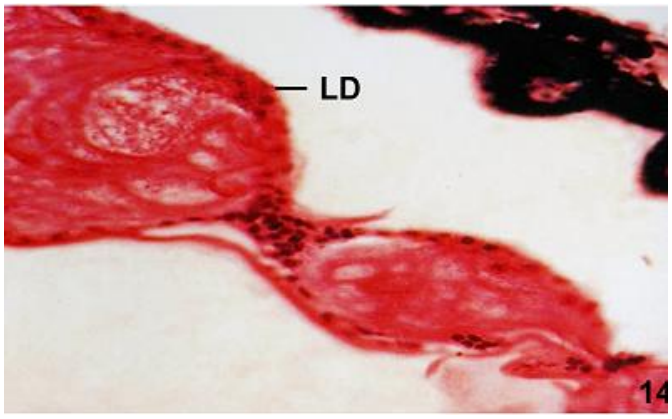

Figure 14. Microphotograph of a section passing through the lentecto-mized eye of 40 days vitamin A treated tadpole showing lentoids (LD) formation (100X). LD, Lentoids.

in four out of 20 animals, lentoid formation was reported. In vitamin A (FTG_3VA.) and ascorbic acid (FTG_3AA) treated animals, lens regeneration occurred in 50 and 45% of animals. The regenerated lenses of these animals too, were found abnormal in shape. Majority of them were smaller in size and variable in shape. Histologically, it has been observed that the origin of lentoid is almost similar to lens formation, that is, they also originated from the dorsal iris in lentectomized iris. In a few cases, lentoids lacked lens epithelium. This was characterized by segregation of individual cells, pycnosis of nuclei and cytoplasmic vacuolization. The lens fibre area showed a well-advanced state of cellular differentiation. In many cases, the shape of lentoid is typically elliptical and elongated. In some lentoids, the lens epithelial cells formed as aggregation of the lens fibre cells. The lens fibre cells showed a stage of differentiation relatively advanced, in comparison to that of regenerated lens of

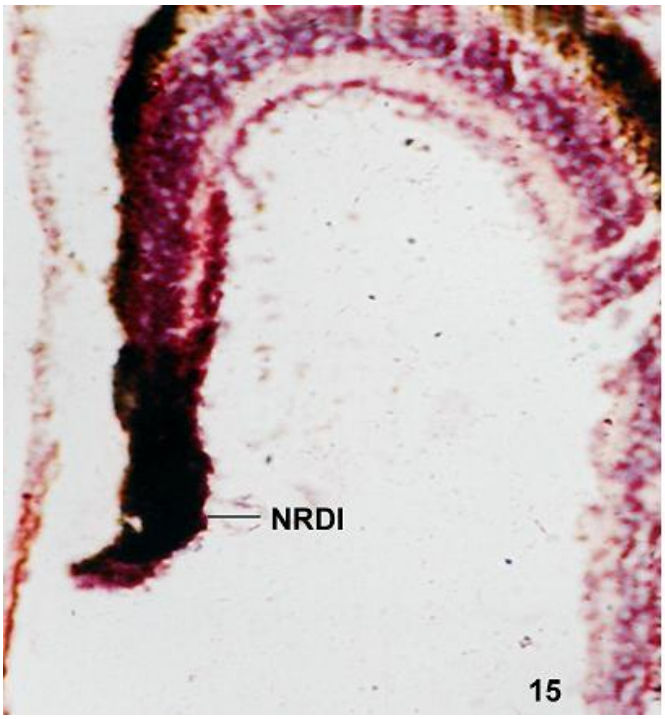

Figure 15. Microphotograph of a section passing through the lentecto-mized eye of 40 days vitamin A treated tadpole showing non regenerating dorsal iris, the both layers of dorsal iris (bilaminar layer) remain closed (50X). NRDI, Non regenerate dorsal iris.

vitamin A treated and ascorbic acid treated animals.

DISCUSSION

The results of the present study showed that both vitamin A and ascorbic acid can induce and accelerate the transdifferentiation of retinal iris PECs into lens *in situ* .In these experimental conditions, vitamin A and ascorbic acid increased the percentage of lens regeneration in all the three developmental stapes of the frog, *R. cyanophlyctis*. As shown in Table 2, the increased percentage of lens regeneration in vitamin A treated animals was 95% in young tadpoles, 80% in mature tadpoles and 50% in froglets. Similar trend was also found in the animals of ascorbic acid treated groups. It was 85% in young tadpoles, 75% in mature tadpoles and 45% in forglets. As shown in Table 2, the percentage of lens regeneration in control group animals was low. Thus, from the present results, it can be concluded that both vitamin A and ascorbic acid are good model for accelerating lens regeneration in the anuran amphibian (*R. cyanophlyctis*). The results also reveal that the regenerative ability in the present species of the frog employed (*R. cyanophlyctis*) declined as the animal ages.

Lens regeneration from non ocular tissue (dorsal iris)

has been well documented in urodele amphibians (Reyer, 1977, 1954; Eguchi and Itoh, 1982; Eguchi, 1988). However, recently it has been observed that the capability of retinal iris PECs to transdifferentiate into lens is not restricted to urodeles amphibians only but is widely conserved in almost all vertebrates (Okada, 2000; Jangir et al., 2000, 2001, 2005; Acharya et al., 2003; Shekhawat et al., 2001). It is well studied that lentectomy stimulates the iris epithelial cells of the newt's eye to undergo DNA synthesis and proliferate (Eisenberg and Yamada, 1966; Yamada and Roesel, 1969; Reyer, 1971). Concomitantly, with these processes, melanosomes disappear from the pigmented epithelial cells of iris and thus undergo dedifferentiation. After completion of the phase of dedifferentiation, some cells retreat from the cell cycle, elongate and proceed to synthesize lens specific proteins and transform into lens fibres (Reyer, 1971; Tsonis et al., 2004). Similar types of regenerative steps of lens regeneration have been observed also in the present treated animals.

The present results confirm the previous report of beneficial influence of vitamin A on lens regeneration. Shekhawat et al. (2001) and Jangir et al. (2005) discovered that vitamin A could induce dedifferentiation in the dorsal iris cells to bring about regeneration of lens. Dedifferentiation is supposed to be prerequisite for regeneration. It is generally an accepted view that if dedifferentiation of an extraordinary degree could be brought, then the cells acquire new developmental potencies. Grigoryan and Mitashov (1991) also studied the effect of retinoic acid on regeneration of the crystalline lens in adult triton and found additional lens from the dorsal iris.

Workers from the same laboratory also observed that vitamin A induced and accelerated lens regeneration in amphibian tadpoles, froglets and chick embryos, swiss albino mice, guinea pigs, rabbits and pigs (Shekhawat et al., 2001; Swami, 1992; Swami and Jangir, 1997, 2000, 1995, 1997; Garg, 1993). The exact mechanism of Vitamin A action is still not well known. However, Chytill and Ong (1984) and Maden (1988) suggested that retinoids enter the cells either via some surface receptors or by lipophilic intercalation through the membranes and then bind to cytoplasmic retinal binding proteins (RABP). Chytill and Ong (1984) and Petkovich et al. (1987), also reported such binding proteins in most of the cells; one specific for retinoic acid, cellular retinoic acid binding protein (CRABP) and other specific for retinal, cellular retinal binding protein (CRBP). The complex is then transported to the nuclei where it ultimately alters the pattern of gene activity. Maden (1988) noticed that the level of CRABP are low in the unamputated axolotl limbs and have risen 3-4 fold in blastemal stage and then decrease again during redifferentiation. This striking co-relation suggests that CRABP may indeed be playing a role in the respecification of positional information. Thus, it can be suggested that retinoids play an important role in the transmission of their legends from the cell membrane to the nucleus where the pattern of gene activity may be altered. Whatever, the mechanism adopted, this can be equally applicable to the observations made by Jangir (1980), Jangir and Niazi (1978) and Niazi et al. (1985, 1989) in limb regeneration of amphibian tadpoles. Vitamin A excess is also found to cause dissolution of cartilage and bone matrix *in vivo* and *in vitro* leading to liberation of healthy, dividing and undifferentiated cells. It is believed to do so by reducing the stability of lysosomal membrane and liberation of hydrolytic enzymes from them (Fell and Thomas, 1960; Dingle et al., 1961; Dingle et al., 1963; Fell and Rinaldini, 1965; Roles, 1969). Polezhaev (1972) have also suggested that if tissue destruction and dedifferentiation is augmented by some means, regenerative ability can be induced or enhanced. In this view, tissue destruction liberates certain biologically active substances possibly protein or nuclei acid in nature, which lead to tissue differentiation and active self-proliferation finally resulting in tissue or organ regeneration.

Similar to present results Jangir et al. (2005), reported that vitamin A induces lens regeneration in mammals also. In support of vitamin A influence on lens regeneration, the report of Tsonis et al. (2000) is much relevant. They studied role of retinoic acid on urodele lens regeneration. They reported that when synthesis of retinoic acid was inhibited by disulfiram or when the function of the retinoid receptors was impaired by using an RAR antagonist, the process of lens regeneration was dramatically affected. In the majority of the cases, lens regeneration was inhibited and lens morphogenesis was disrupted.

Retinoids are found to activate DNA and RNA synthetic activity in several regenerative tissues. In contrast to enhancing DNA and RNA synthetic activity of vitamin A, Yamada and Roesel (1964) studied the effect of actinomycin-D on the lens regenerative system and found that injecting actinomycin-D can inhibit regenerative transformation of the iris cells into the lens. They suggested that the actinomycin-D primarily inhibits the synthesis of RNA which is dependent upon DNA as demonstrated in other system. It is in harmony with the idea that enhancement of RNA synthesis activity observed in iris cells after lens removal is one of the essential steps of the tissue transformation into lens. Thus, for the present observation, that is, enhancement of lens regeneration in vitamin A treated animals, it may be true that vitamin A injection in lentectomized mice could enhanced RNA synthetic activity and hence induction of lens regeneration. This hypothesis is also supported by previous finding of Jangir et al. (1995, 1997) andShekhawat et al. (2001). The work of Harreno Saenz et al. (1994) on lens regeneration in adult newt also supports the present observations. They used antitumor drug 3-nitro benzothiazolo quinolinium chloride (NBQ) and cytotoxic drug doxorubicin for lens regene-

ration in adult newt. They found that NBQ accelerated cell proliferation while doxorubicin inhibits the same and thus NBQ was found to stimulate lens regeneration, independently of the time intervals and the stage of regeneration at which the drug was administered.

The present study and the results of previous studies too show that vitamin A accelerates the rate of mitosis after lentectomy and thus enhanced the dedifferentiation. Mc Devitt et al. (1982, 1990), and Yamada and Mc Devitt (1984) also reported that lenectomy in adult newt initiates the cell cycle and cell division in the dorsal iris cells which are main causative reason for the lens regeneration (Zalik and Scott, 1973, 1990). The pigmented epithelial cells from the dorsal iris dedifferentiate and subsequently transdifferentiate to form the regenerating lens. The restriction of this capability of the pigmented epithelial cells of the dorsal iris, together with the tissue's shorter cell cycle time (compared to ventral iris epithelium) after re-entry into the cell cycle, suggests that the capacity for lens regeneration may be related to mitogenic activity.

It is quite possible that vitamin A affected the cell surface or intercellular space stabilizing factors of dorsal iris epithelial cells and induced the cells for such transdifferentiation. Eguchi (1998) reported in his cell culture study that PECs dissociated from fully grown human eyes readily transdifferentiate into lens phenotype. In addition, Eguchi (1998) speculated that molecules detected in either cell surface or inter cellular space stabilizing the differentiated stage of PECs in the newt and that the loss of these molecules might be one of the key steps of lens regeneration from the iris epithelium.

Results of the present study are also being supported by the finding of Eguchi et al. (1974). In their finding, they observed that under certain conditions, dedifferentiation of extra ordinary cells enable the acquisition of new and higher developmental potencies. In their findings, they observed that under certain conditions, dedifferentiation of extraordinary cells enable them to acquire new and higher developmental potencies.

In the present study, ascorbic acid was found to accelerate dedifferentiation concomitantly increasing the percentage of lens regeneration in comparison to that of untreated control group animals. The exact mechanism of ascorbic acid on lens regeneration is not yet clear and the literature on this Ayurveda drug (with this respect) is very scanty. However, it is reported that a constituent of ascorbic acid have accelerating effect on wound healing (Hellman and Burns, 1958; Jagetia et al., 2003) and enhance proliferation and dedifferentiation of iris pigmented epithelial cells in culture medium. It is reported that ascorbic acid increases the formation of lentoids in culture medium (Kosaka et al., 1998). Good results have being seen in the treatment of diabetic cataract (Suryanarayana et al., 2004), watering of eyes, cough, asthma, bronchitis, peptic ulcer, cardiac disorders. It also improves the mitotic activity in the injured tissues. Thus accelerating effect on lens regeneration in the developmental stages of the animal *R. cyanophlyctis is* a peculiar feature observed in the present study.

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Identification and characterization of milk-clotting proteases produced by two species of mold

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Two strains of fungi were isolated and identified as *Aspergillus tamarii* and *Penicillium pinophilum* which showed good enzymatic activity on casein, 1933.33U and 1822, 21U, respectively. The search for milk clotting enzymes by fermentation at acid pH on culture medium containing whey and, after purification by molecular exclusion chromatography, affinity chromatography and SDS-PAGE helped to find two enzymes having a coagulant activity and approximately molecular weight of 35 and 30 KDa for *Aspergillus tamarii* and *Penicillium pinophilum*, respectively. The optimum pH of activity was 5.5 for both strains and the optimum temperature was 50 and 35°C for *A. tamarii* and *P. pinophilum*, respectively.

Key words: *Aspergillus*, *Penicillium*, milk-clotting enzyme, extraction and purification.

INTRODUCTION

Several proteases of microbial origin are used in production of biological detergents (Lalitha Kumari et al., 2010) as agent for meat tenderization (Chekireb et al., 2009), as milk clotting agent in dairy industries (chymosine and pepsin) and in medical and clinical applications (Llorente et al., 2004), (Sangupta and Dasgupta, 2006). Milk coagulation is a basic step in cheese manufacture and recently, attention has been focused on the production of milk clotting enzymes. Enzymes are aspartic proteases (APs) (EC3.4.23.X) characterized by being more active at acid pH (Timotijević et al., 2004). The strains *Mucor* and *Penicillium* are used mainly as adjuncts in different type of cheese (Fox, 1982), and these recent years other strains like *Rhizopus* (Chun-Chang et al., 2009) and *Aspergillus* (Fezouane-Naimi et al., 2010) prove interesting as a producer of rennet-like enzyme. The goal of this investigation was to identify and characterized milk clotting enzymes produced by two species of mold isolated locally and identify.

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MATERIALS AND METHODS

Reagents

All reagents used were of analytical-reagent grade and solutions prepared using Milli-Q water (Purelab option Q, ELGA). Albumin from bovine serum, calcium chloride, D-tyrosine, ferrous sulfate, folin-Ciocalteux phenol reagent, glucose agar, lactose, magnesium sulfate, peptone, potato-dextrose agar, potassium phosphate monobasic, sodium citrate, trichloreacetic acid (4% (V/V)), Sabouraud 2% and yeast extract were obtained from Sigma-Aldrich (France). Bio-Rad protein assay reagents were purchased from Bio-Rad Laboratories GmbH (Germany).

Culture conditions

Strains of *Aspergillus tamarii* and *Penicillium pinophilum* were seeded on Potato-Dextrose-Agar (PDA) medium at 25°C and monthly transferred. Cultures were stocked as lyophilized spores. Inocula were prepared in 250 mL flask using 50 mL PDA medium. After six days of incubation at 25°C, 50 mL of milliQ water were added. Spores were suspended under agitation with a magnetic stirrer, counted in a cell of malassez and stored at 4°C.

Fermentation medium

The whey (the exploitation of Monique Perruset at Vaugneray, France) was filtered on Whatman No. 1 paper and diluted 1:1 with 0.1 M phosphate buffer, pH 4.0 to generate the basic culture medium. Then, different components were added to this medium: peptone (12.09 g/l), $CaCl_2$ (13.25 g/l) for *A. tamarii* and lactose (12.88 g/l), yeast extract (16.82 g/l), $CaCl_2$ (18.72 g/l) for *P. pinophilum.* Concentrations were previously selected by statistical designs used to optimize the enzymes production. All cultures were performed in 500 mL sterilized flasks (120°C for 20 min) containing 100 mL of culture medium in which 10^6 spores were added. Fermentation was carried out using a horizontal shaker (160 rpm or 100) at 25°C for seven days.

Obtaining of crude protein extract

After fermentation the culture medium is filtered on Whatman's paper to separate mycelium from the extracellular medium containing the desired proteins. Proteins were quantified using the Bradford method (1976), using bovine serum albumin (Sigma-Aldrich) as standard.

Enzyme activity essays

Enzyme activity was measured by the method of Folin-Ciocalteu. Briefly, 0.5 mL of enzyme solution (purified enzyme or culture extract) were added to 0.5 mL of phosphate buffer (0.1 M, pH 4) and 1.5 mL of casein 2.5% in sodium citrate 0.02 M (pH 4), and incubated at 40°C for 60 min. The reaction was then stopped by the addition of trichloro-acetic acid and the amount of released tyrosine was determined (Lenoir et al., 1979). One unit of enzyme activity corresponds to 1 µg of tyrosine released per mL and per hour.

Milk clotting activity (MCA) determination

The determination of milk clotting activity was done according the Berridge clotting time method (1952). To 10 ml of milk in the test tube were added to 0.5 ml enzymatic solution and put in water bath at 35°C. The test tube was submitted to a slight rotation until the flakes of milk appear inside of the wall of the tube test. The time obtained is the mean of two trials. The units of coagulant activity (UAC) or rennet unit (PU) is defined as the amount of enzyme per milliliter of enzyme extract causing flocculation of 10 ml substrate and is calculated as follows:

$$UP = 10 \times V / Tc \times Q$$

Where, UP= Unit Presure (rennet), V = volume of substrate used (ml), Tc = Clotting time (seconds) and Q = Volume coagulant extract (ml).

The coagulant activity can also be expressed as "force coagulant Soxhlet" (F), using the following equation: $F = \frac{UP}{0.0045}$ (Bourdier and Luquet, 1981).

Enzyme purification

Enzymes were first separated according to their size on analytical Sepharose G25 columns (AMERSHAM Biosciences) equilibrated with 25 ml of 50 mM acetate buffer, pH 3.8. After size exclusion chromatography, fractions exhibiting enzymatic activity at pH 4 were purified using affinity column of pepstatin A-agarose (Sigma-Aldrich, France). The column was washed and equilibrated with 50 mM acetate buffer, pH 3.8 and bound proteins eluted with 50 mM Tris/Hcl and 0.5 M NaCl, pH 7. The fractions showing proteolytic activity were pooled and applied to SDS-PAGE electrophoresis. Before any other experiments, the fractions from affinity chromatography purification were concentrated on Amicon ultra-0.5 centrifugal filter units at 14000 rpm for 10 min.

PAGE and molecular mass determination

SDS-polyacrylamide gel electrophoresis of enzyme samples was performed in a Miniprotean II cell (Bio-Rad) on 12% gels (Stacking 5%) according to the procedure of Laemmli (1970). Samples were treated in denaturing conditions with loading buffer and 1 M dithiothreitol (DTT) and boiled 5 min before electrophoresis. Electrophoresis was run at 120 V until the blue dye marker disappeared from the separating gel. Gels were stained with Coomassie Z-blue (Sigma-Aldrich) and un-stained by repeated washing in distillated water. Molecular weight markers used were Euromedex 06P-0111-250 µl (10 to 170 KDa).

Determination of pH and temperature optima

The optimal pH of aspartic proteases was determined by incubating the purified enzyme at 40°C for 1 h in different buffers: acetate buffer (20 mM, pH 3 to 5) and Tris/HCl (20 mM, pH 5 to 8). To determine the optimal temperature, the enzymes of molds were incubated in Tris/Hcl buffer (20 mM, pH 5.5) for 60 min at different temperature: from 25 to 75°C. To determine the thermostability of enzyme activity, the purified enzymes was incubated at different temperatures (45, 50 and 55°C) for *A. tamarii* and (30, 35 and 40°C) for *P. pinophilum*. After each incubation, the enzyme activity was determined as described above.

RESULTS AND DISCUSSION

Purification and characterization of enzymes

Two enzymes with the milk clotting activity were found in

Table 1. Purification data of milk-clotting proteases from two fungi species.

Strain	Purification	Total protein (µg)	Total activity (U.)	Specific activity (U/µg)	Purification fold	Yield (%)
	Crude extract	10309.79	141133.09	13.69	1	100
A. tamarii	Sephadex G -25M	5138.47	78475	15.27	1.11	55.60
	Pepstatine-A agarose	765.77	64931.31	84.79	6.19	46.01
	Crude extract	10089.33	133021.33	13.18	1	100
P. pinophilum	Sephadex G- 25M	9507.52	132860	13.97	1.06	99.87
	Pepstatine-A agarose	3244.12	131562.06	40.55	3.08	98.90

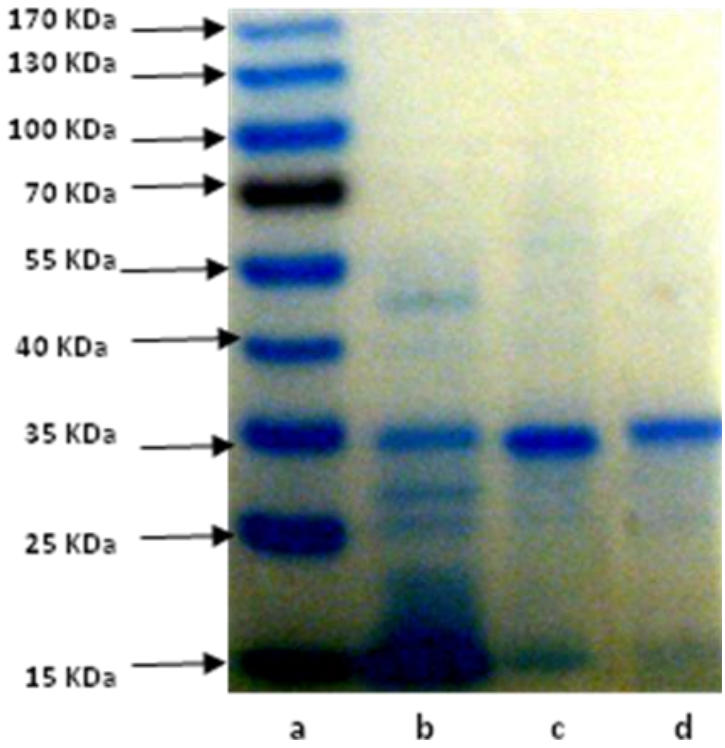

Figure 1. SDS-PAGE protein analysis during the purification of the milk-clotting protease from *Aspergillus tamarii*: a- molecular weight marker, b- crude extract, c- Sephadex G-25M fraction, d- Pepstatin A-agarose fraction.

the crude extract from fungi: *A. tamarii* and *P. pinophilum.* The yields after steps of purification of each strain were: 46.01 and 98.90% for *A. tamarii* and *P. pinophilum*, respectively, with purification folds of 6.19 and 3.08, respectively. The samples treatment with pepstatin A-agarose has eliminated up to 92.57 and 68% of inactive proteins for *A. tamarii* and *P. pinophilum*, respectively (Table 1). The purified enzymes were visualized on gel electrophoresis under denaturing conditions (SDS-PAGE), their subunits have molecular weight of about 35 and 30 KDa for *A. tamarii* and *P. pinophilum*, respectively. The results of gel filtration of the enzymes are shown in Figures 1 and 2.

Optimum pH and temperature

The purified enzymes showed an optimal pH around 5.5 on Tris/HCl and a maximum activity at 50 and 35°C for *A. tamarii and P. pinophilum*, respectively, which indicate that these enzymes might be a acid proteases. Zevaco et al. (1974) in the study of acid protease of *Penicillium roqueforti* observed a high activity of the enzyme on casein at pH 3.5 at 40°C with an optimum at 50°C and stability at pH 3.5 to 5.5.

Hashem (2000) indicates that the clotting enzyme produced by *Penicillium oxalicum* had maximal activity at 65°C and pH 4.0.

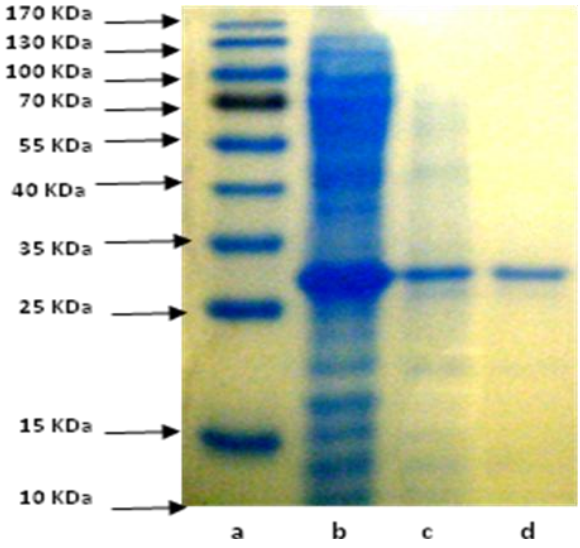

Figure 2. SDS-PAGE protein analysis during the purification of the milk-clotting protease from *Penicillium pinophilum*: a- molecular weight marker, b- crude extract, c- Sephadex G-25M fraction, d- Pepstatin A-agarose fraction.

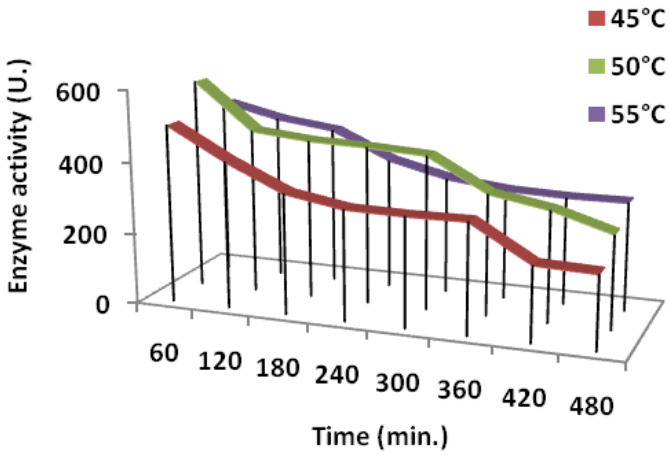

Figure 3. Effect of temperature on the stability of a protease purified from *Aspergillus tamarii*.

Thermal stability

The purified enzyme of *A. tamarii* was studied at various temperatures from 45 to 55°C, the enzyme activity drops significantly in these temperatures (Figure 3), which shows that the enzyme is unstable and its activity decreases from the first hour of incubation, and lost 60, 56 and 40% of the activity at the end of incubation at 45, 50 and 55°C, respectively.

In the case of *P. pinophilum,* the activity of the enzyme at 30°C, increased by 1% during the second hour of incubation and then lost about 13% of activity, every hour, until the end of the incubation, where the enzyme loses more than 57% of activity (Figure 4). At 35°C the enzyme was stable at 2 h, and then lost up to 45% of activity at the end of incubation, at 40°C the enzyme lost activity in the first hour of incubation and showed a $t_{1/2}$ of 4 h.

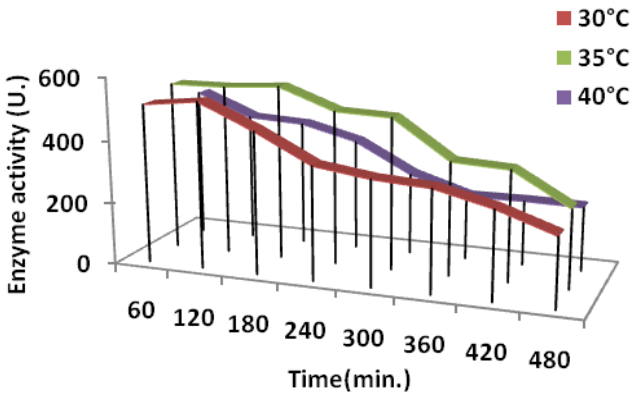

Figure 4. Effect of temperature on the stability of a protease purified from *Penicillium pinophilum.*

In the case of the acid protease of *Penicillium duponti*, Shigenori et al. (1976) showed that the enzyme was stable at pH 3.5 and 6.5 for 24 h of incubation at 30°C, 1 h at 60°C and 1 h at 75°C, pH 3.7.

Milk clotting activity

The crude extract of *A. tamarii* coagulates fresh cow milk at 35°C in 5 min. The enzyme produced coagulates milk in 2 min at 35°C with 184.44 of coagulating force (Table 2). The curd formed appeared clear yellowish serum (Figure 5). The crude extract of *P. pinophilum* coagulates fresh cow's milk in 35 min at 35°C (Figure 6), but the enzyme after purification coagulates milk in 5 min. The curd appears, white to yellowish less firm than that of *A. tamari* with a yellowish, clear and bright serum.

Conclusion

The isolation of fungi producing clotting proteases is important, not only because of the economic interests that represent these molecules but also the medium on which they are grow these strains. The enzymes show a very low apparent coagulating force, despite clotting time; the milk well is below that of rennet, which is explained by the dilution of enzyme solutions obtained after purification.

Conflict of interests

The author(s) have not declared any conflict of interests.

Table 2. Milk-clotting properties of proteases produced by two strains of molds.

Strain	Purification step	Clotting time (min.)	Coagulant activity (UP/ml)	Coagulating force
	Crude extract	5	0.33	73.33
A. tamarii	*Sephadex G -25M*	5	0.33	73.33
	Pepstatine-A agarose	2	0.83	184.44
	Crude extract	35	0.048	10.67
P. pinophilum	*Sephadex G -25M*	10	0.17	37.78
	Pepstatine-A agarose	5	0.33	73.33

Figure 5. Curd obtained by milk coagulation with protease from *A. tamarii*.

Figure 6. Curd obtained by milk coagulation with protease from *P. pinophilum*.

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Full Length Research Paper

Biochemical and cytological analysis of five cultivars of *Cicer* (chickpea)

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During the present study, protein estimation and protein profiling of five cultivars of *Cicer* namely Dhawal, ujjwal, Shubhra, DCP-92-3 and IPC-04-20 have been carried out. The protein content on per gram fresh weight basis was found highest in the Dhawal measuring 35.2± 3.83 mg followed by DCP-92-3 and lowest was found in the IPC-04-20 cultivar. The protein profiling of seed proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) revealed a high polymorphism between the five cultivars. The total number of polypeptide bands recorded was 36, the 4 bands were monomorphic and the rest32 were polymorphic with an average polymorphism of 88.88%. The Jacards similarity ranged from 0.25000 to 0.56000. The similarity index calculated was highest between Ujjwal and IPC-04-20 (37.5%) where as lowest was calculated between Shubhra and IPC-04-20 (22.22%) variety. The unweighted pair group method with arithmetic mean (UPGMA) clustering method revealed two major clusters in the dendrogram that is, cluster 1 and 2, comprising two varieties each. The Shubhra occupies a distinct place as depicted in the dendrogram. Moreover, cytological studies of the five cultivars by calculating the mitotic index were carried out. The mitotic index varied between 4.82 to 10.83% and was found highest in DCP-92-3 while as minimum value was observed in Ujjwal.

Key words: SDS-PAGE, UPGMA dendrogram, mitotic index, chickpea.

INTRODUCTION

The genus *Cicer* include 33 perennial, eight annual, one unspecified wild species as well as the cultivated ones (Van der Maesen, 1987). Chickpea is the second most important cool season pulse crop in the world and is grown in at least 33 countries including central and west Asia, South Europe, Ethiopia, North Africa, North and South America and Australia (Ladizinsky and Adler, 1976; Singh and Ocampo, 1997). It is native to South Europe and is the most important pulse crop of India, commonly grown in Uttar Pradesh, Panjab, Maharashtra, Rajasthan, Bihar and Madhya Pradesh accounting for more than 90% of the total area under it. India is the largest producer of chickpea, accounting for 66% of the world production (FAO, 2004). The average annual yield world wide (0.78 ton/ha) is considered to be somewhat lower than its potential yield (Singh et al., 1994; Sudupak et al., 2002). In India, gram is sown as Rabi crop at the end of the rainy season. Sowing takes place from September to November, and harvesting from February to April. Genotyping of different species is necessary for characterization of different accessions of crop gemplasm, testing varietal purity and registration of newly

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Abbreviations: UPGMA, Unweighted pair group method with arithmetic mean; **SDS-PAGE;** sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Table 1. Five experimental accessions of *Cicer*.

Accession name	Sample name	Source	Seed size
Dhawal	S1	IIPR, Kanpur	Extra bold
Ujjwal	S2	Same	Bold
Shubhra	S3	Same	Bold
DCP-92-3	S4	Same	Small
IPC-04-20	S5	Same	Small

developed cultivars (Chowdhury et al., 2002). Among numerous techniques available for assessing the genetic variability and relatedness, seed storage protein analysis represents a valid alternative to varietal identification (Manella et al., 1999). Seed storage protein profiling based on SDS-PAGE can be employed for various purposes, such as characterization of germplasm (Javid et al., 2004; Iqbal et al., 2005), varietal identfication, biosystematic analysis, determination of phylogenetic relationship between different species (Sammour, 1991; Isemura et al., 2001; Ghafoor et al., 2002). It is a useful tool for studying genetic diversity via sodium dodecyl sulphate polyacrylamide gel electrophoreseis (SDS-PAGE) (Sadia et al., 2009).

Seed storage protein markers are highly polymorphic and environmental influence on their electrophoretic pattern is limited (Gepts et al., 1986; Sadia et al., 2009). Genetic diversity of seed storage proteins has been reported for lima bean (Lioi et al., 1999), *Phaseolus vulgare* (Ferreira et al., 2000) and chickpea (Ghafoor et al., 2003). Phylogenetic relationship among *Cicer* species based on SDS-PAGE data has suggested that *Cicer reticulatum* is the wild progenitor of cultivated chickpea (Ahmad and Slinkard, 1992).

MATERIALS AND METHODS

The germplasm of five accessions of *Cicer* were obtained from Indian Institute of Pulse Research, Kanpur (U.P.) India. During the present study, five accessions were used for biochemical and cytological analysis. The details of five accessions are given in Table 1.

Protein estimation

Cicer being one of the highly proteinaceous crops was subjected to protein estimation in order to know the total protein content. The protein content was measured by using the method of Bradford et al. (1976) and was calculated by using the formula:

$$\text{Protein estimation} = \frac{12 \times \text{O. D.} \times \text{D. F.}}{0.28} \times 1000 \times \text{F. W.}$$

Where, O. D. is Optical Density, D. F. is dilution factor and F. W. is fresh weight.

Protein extraction

For total seed protein extraction from individual seed samples, 1 g seed of each accession was taken and ground into fine powder using pestle and mortar. 500 ml of protein extraction buffer was added to 0.01 g of seed flour and vortexed thoroughly to homogenize. The homogenate samples were centrifuged at 6,000 rpm for 10 min at room temperature. The extracted crude proteins were recovered as clear supernatant and were transferred to a new 1.5 ml eppendorf tubes and stored at 4°C until they were run on the polyacrylamide gel.

Protein profiling

Protein profiling of extracted samples was carried out through SDS-PAGE using 12% polyacrylamide gel. Electrophoresis was carried out at 75 V for 3 h. A protein marker was loaded as standard along with the samples with equal quantities of protein (4 ml) into each well of the gel. The gels were then fixed in solution (10% acetic acid and 40% ethanol) for 15 min with constant shaking and then stained with 0.2% (W/V) Commassie brilliant blue R 250 overnight on an electric shaker using Double Shaker Mixer Model DH-10. Destaining was carried out for a couple of hours followed by gel preservation, scanning and photography.

Protein imaging and data analysis

Gel photographing and documentation were carried out. With regard to variation in protein banding pattern, electrophorogram of each accession was scored for the presence or absence of bands and used to construct a dendrogram by the unweighted pair group mean and arithmetic average method (UPGMA).

Mitotic index

During the present study, the mitotic index was examined. Root tips of each accession were harvested at about 6 a.m., fixed in 3:1 ratio of ethyl alcohol and glacial acetic acid solution for about 6 h and then preserved in 70% alcohol for analysis. The mitotic index was calculated by using the formula:

$$\text{Mitotic index} = \frac{\text{Number of cells in division phase}}{\text{Total number of cells studied}} \times 100$$

RESULTS

Protein estimation

Cicer being a proteinacious crop was subjected to protein estimation. The protein content was found highest in Dhawal (35.2 mg/g F. W.) followed by DCP-92-3 (34.3 mg/g F. W.), Ujjwal (32.9 mg/g F. W.) and Shubhra (31.0

Table 2. Protein content of five accessions of *Cicer.*

Accession name	Protein content (mean ± standard error)
Dhawal	35.2 ± 3.83
Ujjwal	32.9 ± 3.16
Shubhra	31.0 ± 2.33
DCP-92-3	34.3 ± 3.83
IPC-04-20	28.7 ± 1.5

mg/g F. W.); while as the lowest protein content was observed in IPC-04-20 (28.7 mg/g F. W.) (Table 2). The variation might be due to different geographical regions.

Protein profiling

The SDS-PAGE of seed proteins of five genotypes was carried out to investigate the genetic diversity at molecular level. Seed storage protein profiling showed distinct polymorphism in electrophoretic banding patterns and led to detection of 36 polypeptide bands in total. Out of 36 bands, only four were monomorphic and the rest 32 were polymorphic. The average polymorphism was 88.88%. Similar results were also reported by Nisar et al. (2007) in Chickpea. Polymorphism was evident in all seed proteins of diverse molecular weights among all accessions but major diversity was found in low molecular weight region. The molecular weights of peptides ranged from 99 to 40 KD with the presence or absence of particular band. The maximum protein bands generated in case of Dhawal (21) and the minimum (16) in Shubhra. The protein band for highest molecular weight (that is, 99 KD) was generated in all the five accessions while that of lowest molecular weight (that is, 40 KD) was generated in IPC-04-20 (Table 3 and Figure 2). The similarity index calculated was found highest between Ujjwal and IPC-04-20 (that is, 37.5%), and between Dhawal and DCP-92-3 (30.76%) while as minimum similarity was found between Shubhra and IPC-04-20 (22.22%) (Table 4).

The data obtained from SDS-PAGE analysis was used for construction of dendrogram using unweighted pair group mean and arithmetic average (UPGMA) procedure is presented in Figure 1. Five accessions were grouped in two clusters. Cluster I and cluster II, comprising of two accessions each. The cluster analysis revealed that Ujjwal and IPC-04-20 are very close to each other. Accession Shubhra occupies a distinct place as revealed in the dendrogram.

Cytological study

The cytological study was done to find out the mitotic index. The mitotic index varied from 4.82 to 10.83% (Table 5). It was found highest in DCP-92-3 (10.83%) and in Dhawal (8.98%) which revealed that these accessions have highest power of division, while as the minimum mitotic index was found in Ujjwal (4.82%) and Shubhra (7%) which indicates that these have low rate of division. Similarity index of different protein bands was calculated by the formula:

$$S.\ I. = \frac{\text{Total number of similar bands}}{\text{Total number of bands}} \times 100$$

Jaccard's similarity index

Jaccard's similarity index is given in Table 6.

DISCUSSION

Previously morphological and cytological assay procedures were used to estimate existing genetic variability in the crops of commercial importance including legumes (Islam and Shepherd, 1991). These essay procedures though were successful in many cases but were not considered suitable for large scale screening mainly because of limited number of markers and time consuming for the essay procedures. Most recently, protein markers (especially seed storage proteins) are being used for better and more reliable estimation of genetic distances among species/-lines/populations (Weber et al., 2005). Comparison of seed storage proteins have been found to provide no biological basis for separating closely related small and large seeded lentils (Ladizinsky, 1979). Same is the case with chickpea genotypes evaluated in the present study as no difference in seed storage proteins of bold and small seeded genotypes was observed. The protein profiling of seed proteins using SDS-PAGE revealed a high polymorphism between the five cultivars. The total number of polypeptide bands recorded was 36, the 4 bands were monomorphic and the rest 32 were polymorphic with an average polymorphism of 88.88%. The Jacards similarity ranged from 0.25000 to 0.56000. The similarity index calculated was highest between Ujjwal and IPC-04-20 (37.5%); whereas lowest was

Table 3. Presence and absence of bands of different molecular weights in different samples.

Band No.	R. F. value	M. W. K. D.	S1	S2	S3	S4	S5
1	0.01	99	+	+	+	+	+
2	0.02	98	+	+	+	+	+
3	0.03	97	+	+	+	-	+
4	0.05	95	+	-	-	+	+
5	0.06	94	+	+	+	-	+
6	0.07	93	+	+	-	+	+
7	0.08	92	+	-	+	-	-
8	0.10	90	-	+	-	+	+
9	0.11	89	+	-	+	+	-
10	0.12	88	+	-	-	-	-
11	0.13	87	-	+	-	-	+
12	0.15	85	+	+	+	+	+
13	0.16	84	+	+	+	-	-
14	0.17	83	-	-	+	+	-
15	0.18	82	+	+	-	+	-
16	0.20	80	+	-	+	+	-
17	0.21	79	+	+	-	-	-
18	0.22	78	-	-	+	-	-
19	0.23	77	-	+	-	+	+
20	0.25	75	+	+	-	-	+
21	0.27	73	-	+	+	+	+
22	0.30	70	+	-	-	+	-
23	0.31	69	-	+	+	-	+
24	0.33	67	-	-	-	+	-
25	0.35	65	+	+	+	+	+
26	0.36	64	-	+	+	-	-
27	0.37	63	-	+	-	+	-
28	0.38	62	+	-	-	-	+
29	0.41	59	+	-	-	-	-
30	0.42	58	+	-	-	+	-
31	0.45	55	-	-	-	-	+
32	0.46	54	-	-	-	+	-
33	0.48	52	+	+	+	-	+
34	0.50	50	-	+	-	-	-
35	0.52	48	-	-	-	-	+
36	0.60	40	-	-	-	-	+

The symbols (+) and (-) indicate the presence and absence of a band, respectively.

calculated between Shubhra and IPC-04-20 (22.22%) variety. The UPGMA clustering method revealed two major clusters in the dendrogram that is, clusters 1 and 2, comprising two varieties each.

The Shubhra occupies a distinct place as depicted in the dendrogram. Similar results were also reported by Nisar et al. (2007) in Chickpea. The results of present studies are further strengthened by previous finding of Ghafoor et al. (2003), Yasmin et al. (2010), Asghar et al. (2003), Ferreira et al. (2000) and Dasgupta and Singh (2003) who reported high genetic diversity in various legume species using protein profiling.

Conclusion

Genetic diversity is important for improving any crop species. An understanding of the magnitude and patterns of genetic diversity in crop plants has important implications in breeding programs and for conservation of

Table 4. Similarity index of five accessions of *Cicer* using SDS-PAGE.

Sample	Name of samples				
	S1 (%)	S2 (%)	S3 (%)	S4 (%)	S5 (%)
S1	100				
S2	29.16	100			
S3	27.02	27.77	100		
S4	30.76	26.31	23.52	100	
S5	24.39	37.5	22.22	26.31	100

Where, S1 is Dhawal, S2 is Ujjwal, S3 is Shubhra, S4 is DCP-92-2 and S5 is IPC-04-20.

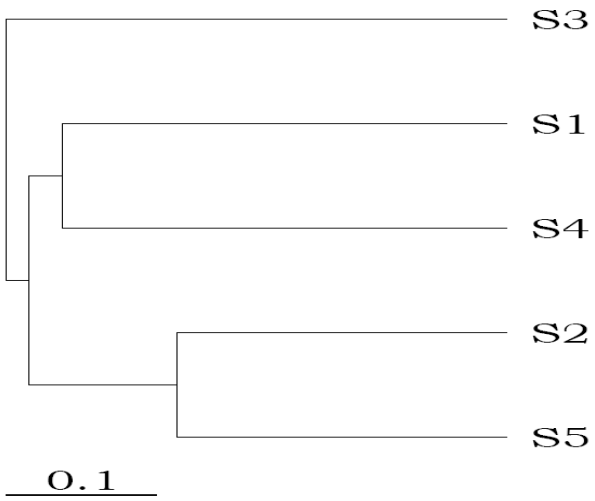

Figure 1. UPGMA dendrogram depicting phylogenetic relationships among five *Cicer* accessions.

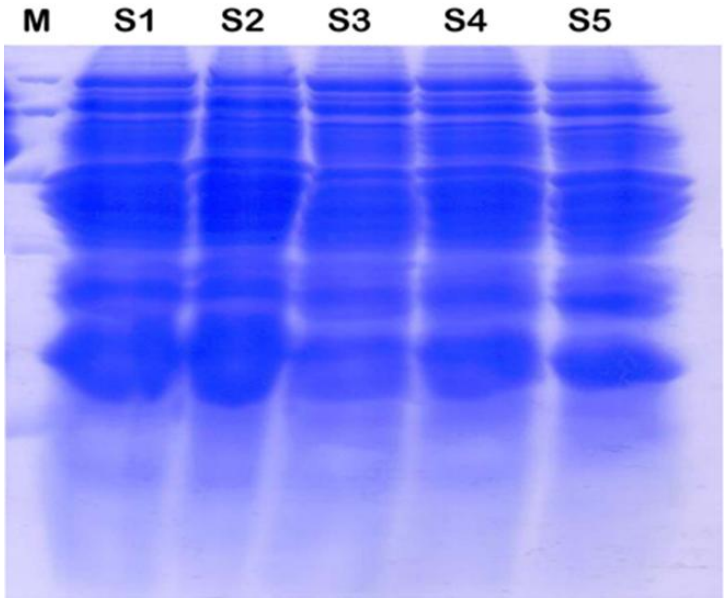

Figure 2. Protein profile of five accessions of *Cicer* produced using SDS-PAGE.

Table 5. Mitotic index of five *Cicer* accessions.

Accessions name	Mitotic index (%)
Dhawal	8.98
Ujjwal	4.82
Shubhra	7
DCP-92-3	10.83
IPC-04-20	7.63

Table 6. Jaccard's similarity index of five *Cicer* accessions.

	S1	S2	S3	S4	S5
S1					
S2	0.41379				
S3	0.37037	0.38462			
S4	0.40741	0.77037	0.32000		
S5	0.33333	0.56000	0.25000	0.33333	

genetic resources. From the result of the study, it is clear that higher amount of genetic diversity of seed storage proteins is present in chickpea genotypes which can be utilized in breeding programs aimed at increasing level of genetic diversity which ultimately will be useful for the development of new improved genotypes of chickpea. Evaluation of genetic diversity and identification of chickpea accessions by SDS-PAGE is easy and early approach and it is also useful for molecular weight analysis of chickpea seed storage proteins. It is concluded from the result that Shubhra and IPC-04-20 are genetically dissimilar; hence, it is recommended that these two accessions should be used for future breeding programs.

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Screening of *Candida boidinii* from *Chemlal* spent olive characterized by higher alkaline-cold adapted lipase production

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A total of 24 lipolytic yeasts were isolated from the spent olive derived from olive fruits of the Algerian variety *Chemlal*. One strain, G5, had the highest lipolytic activity (20 mm) on the tributyrin agar plate. The morphological, biochemical characterization and 18S rDNA gene analysis of the selected strain, confirms that it is *Candida boidinii* KF156789. The production of lipase and biomass were carried out in liquid and solid (spent olive) media. In submerged fermentation, it seemed that the production of enzyme reached its maximum 7.3 U/ml, whereas; the growth cells reached its maximum at 1.9 × 10^8 cell/ml. That can be explained by the assimilation of free fatty acids by this strain after degradation of olive oil by the enzyme. The production of lipase and biomass, in solid state fermentation, gave the maximum yield for cell growth (1.3 × 10^9 cell/ml), while lipolytic activity reached 4.8 U/g. The highest activity of the studied enzyme was at pH 7.0 and 37°C. The enzyme maintained more than 90% of its activity at pH 8.0-9.0 and 70% at temperature range of 4-40°C; it was concluded that the lipase from *C. boidinii* KF156789 has the potential to be an alkaline cold-adapted enzyme.

Key words: *Candida boidinii* KF156789, higher lipase, alkaline cold-adapted lipase, *chemlal* spent olive, solid state fermentation.

INTRODUCTION

Lipases (EC 3.1.1.3) constitute the most important group of biocatalysts for biotechnological applications. They have immense applications in various fields, such as food, pharmaceutical, cosmetic, agrochemical, feedstock, detergent, textile, biodiesel and oil processing industries (Treichel et al., 2009). These enzymes are ubiquitous in nature and are widely distributed in plants, animals and microorganisms. According to Vakhlu and Kour (2006), the main lipolytic yeasts isolated from terrestrial environment are: *Candida rugosa, Candida tropicalis, Candida antarctica, Candida cylindracea, Candida parapsilopsis, Candida deformans, Candida curvata,*

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Candida valida, Yarrowia lipolytica, Rhodotorula glutinis, Rhodotorula pilimornae, Pichia bispora, Pichia mexicana, Pichia sivicola, Pichia xylosa, Pichia burtonii, Saccharomycopsis crataegenesis, Torulaspora globosa, and Trichosporon asteroids. Other strains, such as *Candida boidinii* 1638, have lipase negative as described by Ciafardini et al. (2006). After that, Rodriguez-Gomez et al. (2010, 2011) remarked that all strains of *C. boidinii*, isolated during process of fermentation of olive table, can produce lipase.

In this work, *C. boidinii* was isolated from local oily environment as spent olive; because it may provide a good environment for lipolytic strains to flourish and for isolation of lipase producing microorganisms (Ohimain et al., 2012). In fact, Olive cultivation (*Oleaeuropea L*.) and olive oil production are important in Algeria, where several varieties of olives trees are grown. The most important one is *Oleae uropea L*. variety of *Chemlal*, where it represents the most widespread in Algeria (more than 50% of the olive groves) (Tamendjari et al., 2009). Therefore, the objective of this study was to isolate the highest lipolytic yeast from olive residue (spent olive, *Chemlal* variety), collected from manufacturers of Skikda (East of Algeria). One isolated strain *Candida boidinii* KF156789 was found to be able to produce highly extracellular lipase, which was detected qualitatively and quantitatively in liquid and solid media (as shown in the following sections). To our knowledge, this is the first report about isolation and characterization of highest lipolytic yeast, *Candida boidinii* KF156789, from spent olive of *Chemlal* variety. In addition, it was important to investigate the production of lipase on the spent olive *Chemlal* (SOC).

MATERIALS AND METHODS

Site description and sample collection

Samples of spent olive (*Chemlal variety*) used in this study were collected from manufacturers of olive oil located in Skikda, in the North East of Algeria, known as an important olive oil producers. The samples were collected during pressing of olive oil. Samples were kept in sterile bottle containers.

Isolation of yeasts from spent olive

The isolation process was performed by serial dilution of samples on Sabouraud Chloramphenicol medium (Pasteur institute, Algeria).The purification of strains was established on yeast extract, peptone, dextrose (YPD) agar medium, incubated at 28°C and examined during five days (Harju et al., 2004). Colonies with distinct morphological differences such as colour, shape, and size were picked and purified by streaking at least three times on YPD agar; the isolates were stored at 4°C and sub-cultured for 15 days intervals.

Screening of lipolytic yeasts

The colonies of isolated yeasts were examined on modified tributyrin plate agar and clear zones around the colonies indicate production of lipase (Cardenas et al., 2001). This medium composed of (g/l): 10 peptone; 12 yeast extract; 30 tributyrin and 10 agars (pH 6.2). The Petri dishes were incubated at 28°C for 3 days. The diameter (d) of the colonies and the diameter (D) of total hydrolytic halos including the colonies were determined. The strain that yielded higher halos (D-d) was selected as potential microorganism for lipase production using tributyrin as substrate (Griebeler et al., 2009; Hassan et al., 2009).

Morphological characteristics

The cultural morphology of the selected isolate was examined on YPD liquid and agar plates after incubation at 28°C for 3 days. The diameter colony, morphology and texture on plates were analyzed. The formation of filaments was characterized as described by Guiraud (1998).

Physiological and biochemical approach

Yeasts were identified using the conventional methods described by Wickerham (1951), Van der and Yarrow (1984), Deak and Beuchat (1996) and also by using the API 20C AUX test strips (Bio-Merieux, Belgium). The tests included the fermentation of sugars, liquid assimilation of carbon compounds, liquid assimilation of nitrogen compounds, growth at 30 and 37°C, growth in media containing 16% NaCl, resistance to 0.01% Cycloheximide and detection of urease activity.

Molecular approach

The total genomic DNA of the yeast strain was isolated, purified and amplified using the methods as described by Promega. The used primers for amplification of 18S ribosomal DNA in selected yeast were 5'-CTT-TCG-ATG-GTA-GTG-TAT-TGG-ACT-AC-3' and 5'-TGA-TCC-TTC-TGC-AGG-TTC-ACC- TAC-3'. The sequencing of the PCR products was performed in Progenus (Belgium). The sequences were corrected by the Bio-edit program and deposited in Genbank data base. The DNA sequences were compared to those previously published in Genbank using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

Production of lipase in submerged medium (SmF)

The yeast strain grew in the liquid medium YPD for 24 h at 30°C, for reactivation of strain. 10 ml of this culture were used as the inoculum for production of enzyme. The lipase enzyme was produced in flasks as described by Destain et al. (1997). It was cultivated in 250 ml Erlen meyer flasks containing 100 ml medium composed of (g/l): 10 glucose, 30 whey powder, 8 $(NH_4)_2$ SO_4, 10 corn steep liquor and 5 olive oil (extra virgin) (pH 6.5). The production broth (100 ml) was incubated at 30°C under shaking (120 rpm, 120 h) conditions. The cell numbers were estimated and lipase activity was measured.

Production of lipase in solid state fermentation (SSF)

The SOC was used as a natural substrate for the solid state fermentation. The used method was followed as described by Moftah et al. (2011). For the fermentation, spent olive samples were taken and dried in a general purpose oven for 1 h at 105 ± 5°C. Next, the sample was grounded and sieved to provide particle sizes >800 µm. SSF of spent olive was carried out in 150 ml Erlen meyer

Table 1. Hydrolytic activity on agar plates after three days.

Strain	Tributyrin use	Strain	Tributyrin use
G4	++	**G5**	**++++**
G9	++	G13	++
Gb1	++	Gb2	++
Gb3	+	Gb4	+
Gb5	++	Gb6	+
Glb1	++	Glb2	+
Glb3	+++	Glb33	++
Glb7	++	GG1	+
GG2	++	GG3	++
GG4	+	GG5	+
Gg1	-	Gg2	++
Gg3	++	Gg4	++
Gg6	+		

Symbols are related to the diameter of halos: + < 5 mm; ++ between 5 and 10 mm; +++ between 10 and 15 mm; ++++ is 20 mm.

flasks to study the effects of the selected strain in its origin media for lipase activity and biomass yield. The media in flasks (10 g of dry substrate) were autoclaved at 121°C for 20 min. One milliliter of autoclaved distilled water was added to this autoclaved preparation before inoculation. Further, SSF was carried out by inoculating 10 g of SOC (moisture content adjusted to 90%) with 1 ml of inoculum (approximately 1.7×10^7 cells) followed by incubation at 30°C. The basic medium was enriched with YPD medium supplemented with olive oil (0.5%) and tween 80 (0.1%). The water added with the inoculum was also considered in moisture correction. The pH of the prepared medium was adjusted to 6.5. The samples are aseptically withdrawn at various time intervals (one, two, three, four and five days). The cell numbers were estimated and lipase activity was measured.

Extraction of crude enzyme from SSF

To extract the enzyme, a known quantity of the fermented media was mixed with distilled water (1:4, w/w) by shaking on a rotary shaker (180 rpm, 30 min, 30°C); then, the whole contents were centrifuged at 8,000 rpm for 10 min (4°C), and the supernatant is used as a crude enzyme extract (Moftah et al., 2011).

Lipase assay

After centrifugation of samples at 8000 rpm for 20 min at 4°C, the supernatant was used as the source of extracellular crude enzyme. Lipase activity was assayed by titration using olive oil as substrate as described by Mafakher et al. (2010). The assay mixture consisted of 5 ml of emulsion (olive oil 15 ml, NaCl 30 ml and completed until 200 ml with polyvinyl alcohol), 4 ml of phosphate buffer (0.1 M; pH 7.0) and 1 ml of the crude enzyme. The preparation was incubated for 15 min at 37°C. The reaction was terminated by adding 20 ml stop solution (1:1 norvanol/acetone), and the amount of liberated fatty acids during incubation was tittered with 0.05 N NaOH in the presence of phenolphthalein as an indicator. The enzyme assay was analyzed in triplicate. One unit of lipase activity was defined as the amount of enzyme that librated 1 µmole of Free Fatty Acids (FFAs) per ml under the assay conditions.

Cell growth

Determination of yeast cell growth was performed by spreading suitably diluted cell suspensions on YPD plates and counting the yeast cell colonies after 48 h of incubation at 30°C.

Partial characterization of lipase: Effects of pH and temperature

Lipases are known to be diversified in their catalytic properties, and therefore, it is important to characterize them. The partial characterization of crude enzyme obtained by submerged fermentation tested at the large range of pH and temperature. pH optimum was determined by carrying out the enzyme assay at 37°C by titrimetric method. The phosphate buffer (0.1 M) was used at pH 5-8, adding acetic acid (0.1 M) at pH 3-4 and NaOH (0.1 M) at pH 9. The temperature optimum of the enzyme was evaluated by measuring the lipase activity at different temperatures (4-70°C) in 0.1 M phosphate buffer pH 7.0. The enzyme assay was analyzed in triplicate.

RESULTS

Screening of highest lipolytic yeast from the SOC

In this preliminary study, 25 colonies with morphology typical of yeast were isolated from the SOC samples that can utilize glucose as the sole carbon source. Most of the isolated strains (24 strains) grew on medium contains tributyrin as carbon source; which prove that the majority of isolates have a lipase activity. Table 1 shows the average diameter of extracellular lipolytic activities halos zone of the isolated yeasts on tributyrin agar plates. The strain G5 showed better lipolytic activity than the others strains. The diameter of its halos zone reached 20 mm.

Morphological characteristics of the selected strain

The colony types and cells morphology of selected strain which were investigated in this study are depicted in Figure 1. This strain has aerobic growth and formed white, butyrous colonies with convex elevation and entire margin; the diameter of colonies is around 2-3 mm (Figure 1a and b). In microscopically appearance, it has oval and cylindrical multilateral budding cells (Figure 1d). The diameter of cells is 3 µm and 8 µm of length, after 48 h of culture in YPD broth medium (Figure 1c). The filamentation test showed that the strain develops a pseudo-hyphae consisting of long branched chains of cells with blastoconidia (Figure 1e and f).

Physiological characterization

The selected strain (G5) was identified by biochemical and physiological tests (Table 2). This identification was carried out by using API 20C AUX (Bio-Merieux, Belgium) and other residual biochemical tests. API test was

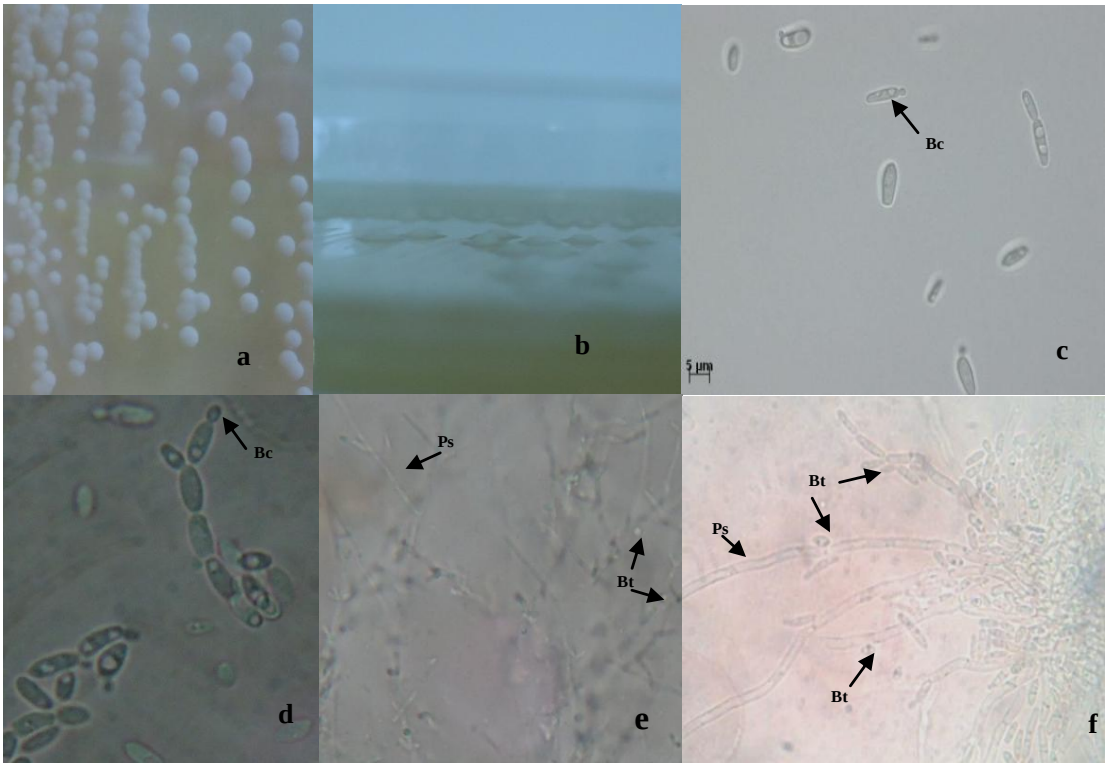

Figure 1. Morphological aspect of G5 strainisolated from the Algerian spent olive *Chemlal* variety: **a,** culture on YPD agar after 48 h at 30°C; **b,** convex elevation of colony on YPD after 48h at 30°C; **c** and **d,** microscopically arrangement of the cell in wet mount (G×100); **e** and **f,** filamentation test on PDA medium after 48h (G×100); **Ps**: pseudo-hyphae, **Bt**: blastoconidia, **Bc**: budding cell; (Photos taken in laboratory of Mycology, Biotechnology and Microbial Activity, Constantine, Algeria and in Wallon Centre of Industrial Biology, Gembloux Agro Biotech, Belgium).

classified G5 as *Candida boidinii*, with probability of 0.99; considered as a good score. In addition, the results of the rest biochemical test shows that *C. boidinii*i was able to ferment just glucose and fructose. However, the strain can assimilate a diversity of carbon compounds such as glucose, fructose, xylose, glycerol, adonitol, xylitol, sorbitol, trehalose and mannitol but not the other used substrates. Otherwise, other tests had revealed that the strain can use the potassium nitrate and had exhibited a good growth at 30°C but not at 37°C.

Molecular identification

In order to deepen more in identification study of the selected strain, the regions of 18 S ribosomal DNA was amplified, and PCR products were analyzed. The sequences were compared with available DNA sequence databases using BLAST program (Figure 2). After comparing the sequences to the GenBank database, it was demonstrated clearly in the phylogenetic tree that the strain (G5) was identified as *C. boidinii* KF156789 with a similarity of about 100% of the 813-nucleotide sequence.

The kinetics of enzyme production and cell growth in SmF

The results concerning the production of extracellular lipase and biomass of *C. boidinii* KF156789, in liquid medium were depicted in Figure 3. The results shows that the *C. boidinii* KF156789 produces the maximum activity of extracellular lipase (7.3 U/ml) after 26 h of fermentation; Whereas, the maximum growth cells (1.9×10^{08} cell/ml) was reached after 72 h of fermentation. At the end of fermentation, the lipase activity diminished gradually, leading to the gradual reduction in the growth of *C. boidinii* KF156789.

The kinetics of enzyme production and cell growth in SSF

Using basal medium formulation, the kinetics of the production of lipase and biomass, were investigated (Figure 4). It appears that the production of lipase and biomass increased steadily with the cultivation time. The lipase activity and biomass yield reached its maximum, 4.8 U/g and 1.3×10^{09} CFU/g, respectively, at the end of cultivation.

Table 2. Biochemical and physiological tests of high lipolytic yeast G5 isolated from the Algerian spent olive *Chemlal* variety.

Substrate	Fermentation of sugars	Assimilation of carbon compounds	Other tests
Glucose	+	+	
Fructose	+	+	
Galactose	-	-	
Xylose	-	+	
L- Arabinose		-	
Saccharose	-		
Lactose	-		
Maltose	-	-	
Starch	-	-	
Glycerol		+	
calcium 2-Keto-Gluconate		-	
Adonitol		+	
Xylitol		+	
Inositol		-	
D-Sorbitol		+	
Methyl-αD-Glucopyranoside		-	
N-Acetyl-Glucosamine		-	
D-Cellobiose		-	
D-Trehalose		-	
D-Melezitose		-	
D-Raffinose		-	
Mannitol		+	
Potassium nitrate			+
16%NaCl			-
Cycloheximide 0,01%			-
Urease			-
Growth at 30°C			+
Growth at 37°C			-

Partial enzyme characterization: Effects of pH and temperature

The influence of pH and temperature in the enzyme activity is presented in Figures 5 and 6, respectively. The highest activity (7 U/ml) was found at pH 7.0 and at 37°C. The enzyme was most active in pH range between 7.0 and 9.0, that it showed more than 90% of its activity at pH 9. In addition, the enzyme kept just 50% of its activity at pH 4. On other hand, the lipase kept about 90% of its activity between 30 and 40°C, noted that this enzyme present 70% of its activity at 4°C.

DISCUSSION

Algeria has about 32 million olive trees spread over 300,000 hectares and produce 4.7 million quintals of olives. The local variety *Olea europea L*. *Chemlal* exists with highest values 80% in the territories reserved to oil olive trees, especially, in the north eastern of Algeria. The spent olive is a by-product of oil extraction; this residue consists of the solid constituents of the skins, pulp, seeds and fragments of core (Moussaoui et al., 2008). In this work, several yeasts were isolated from Algerian spent olive (*Chemlal*) collected from olive oil mills in Skikda (Eastern of Algeria). One strain (G5) of them was selected as the highest extracellular producer of lipase. The combination of morphological, biochemical and molecular properties can lead to identifying G5 as *C. boidinii* KF156789.

Usually, the cited strain was isolated from soil, but, several authors have isolated it during industrial fermentation of table olives (Bautista-Gallego et al., 2011; Rodriguez-Gomez et al., 2011). In addition, *C. boidinii* was known in literature as methylotrophic yeast (Janatova, 1992), however, recent investigations have shown its capacity of lipase production (Rodriguez-Gomez et al., 2010). The production of lipase was carried out on two different systems of fermentation SmF and SSF. As shown in Figure 3, the surge of exponential growth, which could have been the result of the lipase

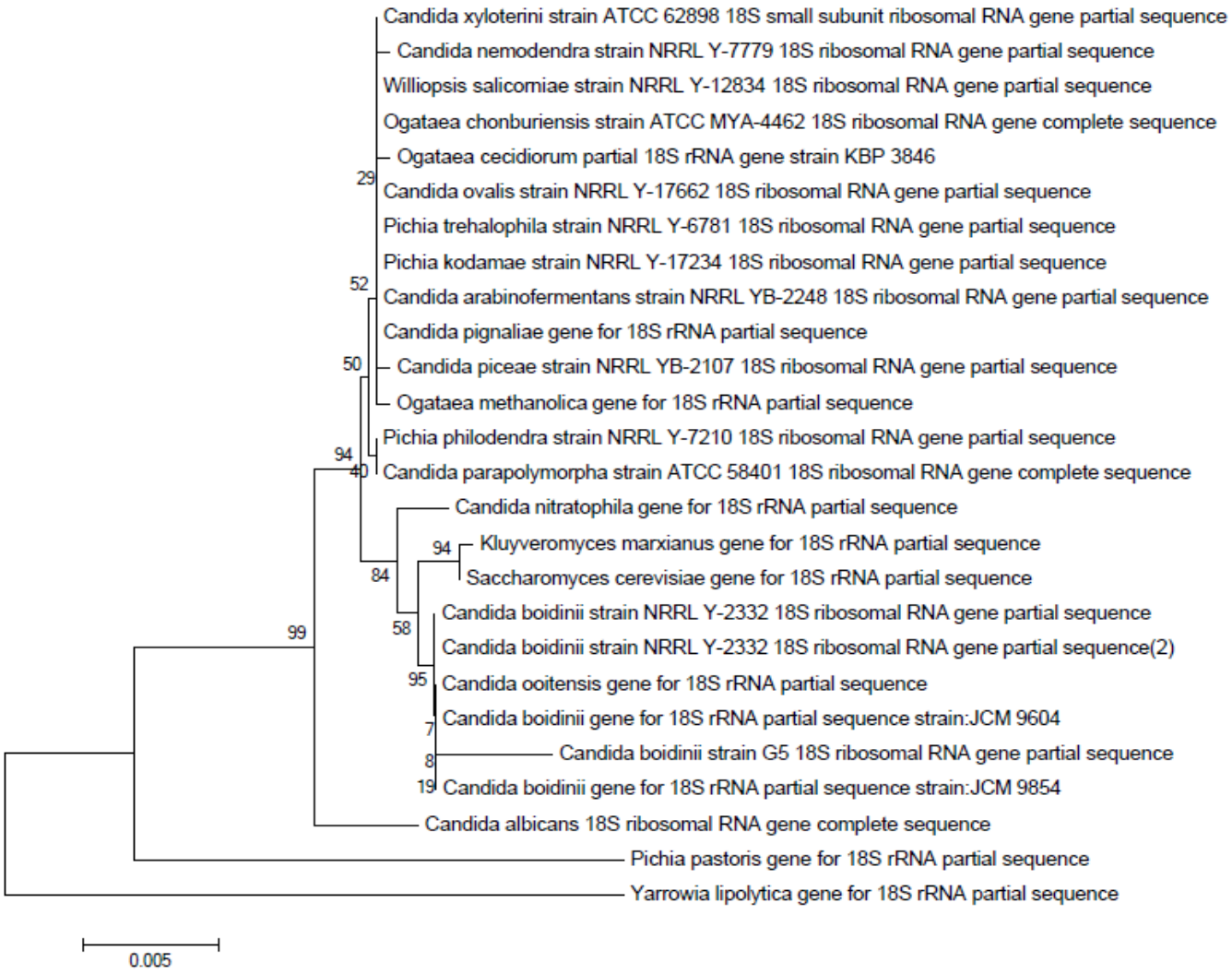

Figure 2. Evolutionary tree of the selected strain (*C. boidinii*) based on 18S rDNA gene sequences obtained in this study.

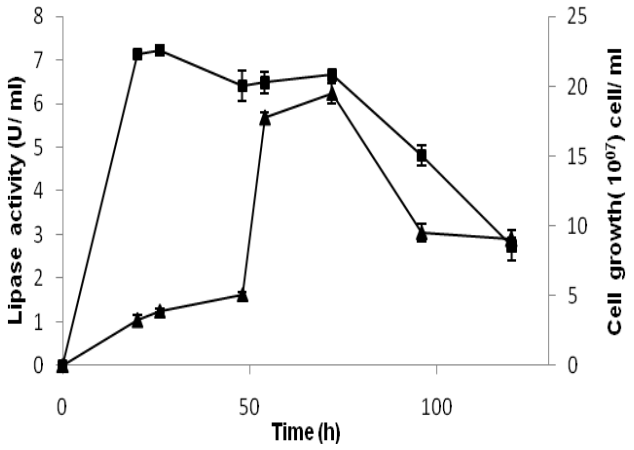

Figure 3. Production of lipase and biomass in SmF for 120 h at 30°C: (▲) cell growth; (■) lipase activity.

activity, breaking down of olive oil, leading to the creation of fatty acids that could be assimilated easily by the used strain after 26 h of incubation. The extra virgin olive oil was found to be the most suitable enhanced source of carbon and considered as inducer for lipase production (Mobarak-Qamsari et al., 2011).

In addition, it was important to investigate the production of lipase and biomass of the cited strain in its origin medium (SOC). Lipase production in SSF process seems to have the most cell growth and low lipolytic production (Figure 4), in comparison with SmF. The decay of extracellular lipase activity during the later period of both cultures may be related to the effect of pH and temperature, or it was probably caused by proteo-lysis. Several recent studies reported that SSF has many preferences to SmF for microbial enzyme pro-duction including: high yield and productivities, enzyme stability

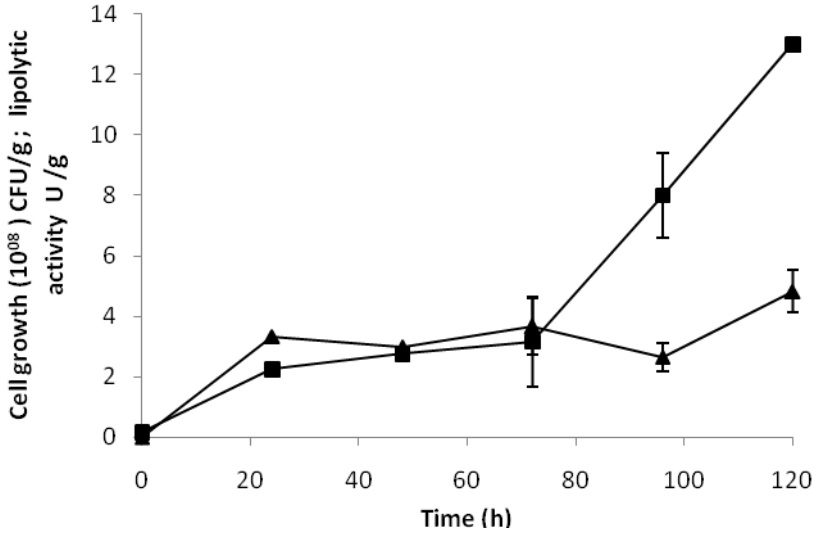

Figure 4. Production of lipase and biomass in SSF for 120h at 30°C; (▲) Lipolytic activity; (■) Cell viability.

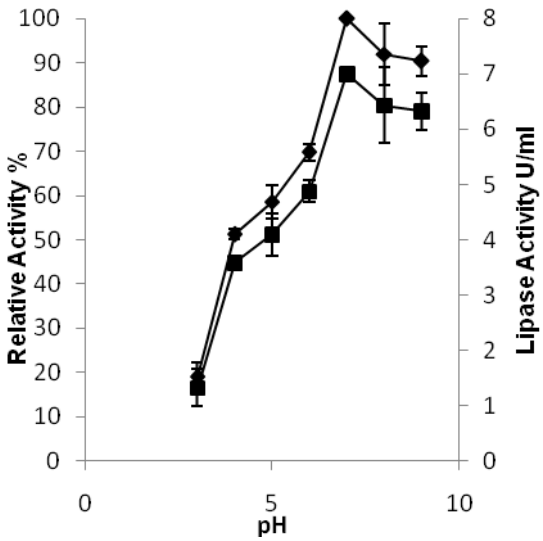

Figure 5. Effect of pH on lipase activity: (■) lipase activity; (♦) relative activity. The experiments were performed in triplicate and bars represent the standard deviation.

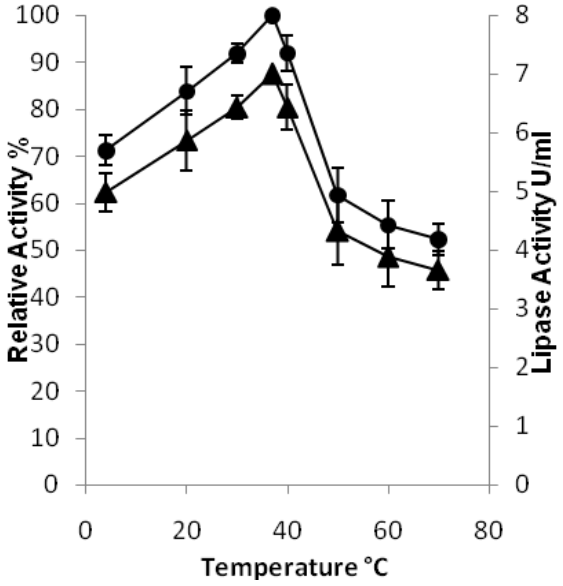

Figure 6. Effect of temperature on lipase activity: (▲) lipase activity; (●) relative activity. The experiments were performed in triplicate and bars represent the standard deviation.

and low cost (Hosseinpoura et al., 2012); whereas in other research, in particular, for the production of laccase obtained by the fungus *Trametes versicolor* ATCC200801, the production in submerged fermentation is seen maximum benefit of production facilities and the minimum cost (Demir et al., 2011). Other investigations about the production of lipase on the SOC are still needed. It was interesting also to know that lipase from *C. boidinii* KF156789 is active in a pH range of 7-9, with a maximum lipase activity at pH 7, and between temperatures of 30-40°C with an optimum temperature for the lipase activity at 37°C. These results are similar to that reported for lipase from *Y. lipolytica, A. johnsonii LP28* and *Aeromonas sp. LPB 4* (Lee et al., 2003; Brigida et al., 2007; Wang et al., 2010). So, lipase from *C. boidinii* KF156789 may be good in industrial applications (as industry of detergent), at different range of temperature and alkaline pH.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Jabuticaba [*Pliniajaboticaba* (Vell.) Berg] skins decrease lipid peroxidation: Hepatoprotective and antihyperlipidemic effects

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The effect of Jabuticaba [*Plinia jaboticaba* (Vell.) Berg] Skin Flour (JSF) was studied on peroxidation, plasma and hepatic lipid profiles of female rats, as well as quantification and characterization of phenolic compounds. The animals were divided into four groups of eight rats. The groups received 0 (control); 0.5; 1.5 and 3.0 g JSF per 100 g diet. The diet with 3.0% JSF increased the HDL level by 20.23% compared to the control. The groups that received JSF had lower AST and ALT activities, when compared to the control group. There was a decrease of macro vesicular steatosis in the liver of animals fed the diet supplemented with 3.0% JSF. The diets containing 1.5% and 3.0% JSF reduced lipid peroxidation in the liver by about 50%. JSF was effective in protecting against dyslipidemia, because it increased the serum level of HDL cholesterol, showed a good antioxidant activity and demonstrated hepatoprotective effect.

Key words: *Plinia jaboticaba*, phenolic compounds, HDL cholesterol, antioxidant action, HPLC.

INTRODUCTION

Jabuticaba [*Plinia jaboticaba* (Vell.) *Berg*] is a typical Brazilian fruit that features pleasant sensory characteristics, with its soft, juicy and bittersweet pulp (Danner et al., 2006). When consumed fresh, the skin is discarded as waste. The fruit has high water content and sugars, which makes it highly perishable, with a short life after harvest. Aiming to minimize significant economic losses, several studies have been conducted for a better

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Abbreviations: ABTS, 2,2′-Azinobis-(3-etilbenzotiazolin-6-sulphonic) acid; **ADC,** average daily consumption; **ADG,** average daily weight gain; **ALT,** alanine aminotransferase; **AOAC,** association of official analytical chemists; **AST,** aspartate aminotransferase; **DM,** dry matter; **FER,** feed efficiency ratio; **GGT,** gamma glutamyl transferase; **HDLc,** high-density lipoprotein cholesterol; **HPLC,** high-performance liquid chromatography; **JSF,** jabuticaba skin flour; **LDLc,** low-density lipoprotein cholesterol; **MDA,** malondialdehyde acid; **NFE,** nitrogen-free extract; **TBARS,** thiobarbituric acid reactive substances.

use of the fruits (Alves et al., 2013; Asquieri et al., 2004). Jabuticaba skin contains bioactive compounds with potential to promote health benefits. Research has shown that they have an antiproliferative effect against leukemia and prostate cancer cells (Leite-Legatti et al., 2012). Jabuticaba Skin Flour (JSF) is rich in soluble and insoluble fiber [27.03 and 6.77 g 100 100 g^{-1} dry matter (DM), respectively] and has a high content of total phenolic compounds (11.99 g 100 g^{-1} DM), including anthocyanins (2.06 g 100 g^{-1} DM), responsible for its characteristic color (Lima et al., 2008, 2011b). High-performance liquid chromatography (HPLC) analyses detected the anthocyanins cyanidin-3-glucoside and delphinidin-3-glucoside in JSF (Leite-Legatti et al., 2012). It presents antioxidant potential, probably due to the high content of phenolic compounds (Lima et al., 2008, 2011a). The consumption of fiber and phenolic compounds can beneficially affect the population health, and are known to be important in the prevention and treatment of diseases.

Afonso et al. (2013) suggested that phenolic compounds attenuate oxidative stress and reduce cholesterol levels in the blood of rats. Food that is rich in phenolic compounds, such as green tea and blueberry, show an inhibitory effect on hepatic steatosis (Park et al., 2011; Liu et al., 2011). The occurrence of non-alcoholic hepatic steatosis has been associated with the excess accumulation of lipids in the liver, liver injury and dyslipidemia. Hepatic steatosis causes elevations in serum aminotransferases, and the analysis of these enzymes is one of the forms to diagnose its occurrence. Diets containing antioxidants have been used for the prevention and as a strategy to limit the accumulation of lipids and liver damage (Park et al., 2011; Bruno et al., 2008). Jabuticaba skins have chemical characteristics that demonstrate their potential as functional and/or nutraceutical food; however, studies on possible applications in health promotion are scarce. In this context, the objective of this study was to analyze the effect of JSF on peroxidation, plasma and hepatic lipid profiles of female rats, and quantification and characterization of its phenolic compounds.

MATERIALS AND METHODS

Preparation of the jabuticaba skin flour (JSF)

P. jaboticaba (Vell.) Berg fruits, Sabará genotype, were hand-picked on São José do Ismeril Farm, in the municipality of Coqueiral, MG, Brazil, transported to the laboratory, where they were selected, washed in tap water, sanitized with sodium hypochlorite solution (200 mg kg^{-1}), by a 10 min immersion; they were then squeezed and the skins were weighed and separated into three lots of approximately 2.9 kg. The jabuticaba skins were dried in a food dehydrator, in mesh metallic material baskets, at a temperature of 45°C, with a 1 m s^{-1} air flow over a period of 36 h. The skins were then ground and the resulting JSF was packaged in hermetically sealed flasks in three replicates, wrapped in aluminum foil, stored at room temperature and subjected to analysis. This JSF was classified as fine grain.

Proximate composition

The proximate composition (moisture, ether extract, crude protein (N X 6.25), ash, dietary fiber and nitrogen-free extract) was performed, based on the methodology described by the Association of Official Analytical Chemists (AOAC, 2005).

Chromatographic study of phenolic compounds

The extraction of phenolic compounds was performed using 50% methanol in the ratio 1:25 (w/v). Chromatographic analyses were performed using an Agilent HPLC equipment model 1100, and the best response was obtained at a wavelength of 280 nm. The extract of phenolic compounds and the standards were injected, in three replicates, into an Ascentis C_{18} column (250 mm × 4.6 mm × 5 µm), attached to a Supelguard Ascentis C_{18} pre-column (20 mm × 4.0 mm × 5 µm). The mobile phase was composed of the solutions: 2% acetic acid (A) and methanol:water:acetic acid (70:28:2 v/v/v) (B). The flux used in all analyses was 1.00 ml min^{-1}; the injection volume was 20 µL. Analyses were performed in a total time of 65 min at 15°C in a gradient-type system: 100% solvent A for 5 min, 70% solvent A for 20 min, 60% solvent A for 18 min, 55% solvent A for 7 min, 0% solvent A for 10 min. Until the end of the run, solvent A was increased to 100%, in order to balance the column. Addition of standards to the extracts was also used as an identification parameter. Quantitation was performed using external standardization with concentrations of standard stock solutions: Gallic, p-coumaric, ferulic, ellagic, 3,4-dihydroxybenzoic, syringic and salicylic acids, as well as the gallocatechin, catechin, epigallocatechin gallate and resveratrol (Sigma-Aldrich - St. Louis, MO, USA).

Vanillic acid and m-and o-cumaric acids (Fluka - St. Louis, MO, USA). Stock standard solutions were prepared in dimethylsulfoxide and/or methanol (Merck). Each solution was injected three times on the HPLC system, with the purpose of obtaining concentration means and retention times.

Animals and treatments

All procedures were performed in accordance with the ethical principles in animal experimentation, adopted by the Ethics Committee on Animal Use of the Universidade Federal de Lavras (Protocol 009/11, approved on 09/01/2011). Thirty two female Fischer rats were used, with a body weight of approximately 140 g, divided into four groups with eight animals in each group. The animals were kept in individual cages, in a room with a temperature of 25 ± 3°C (light/dark cycle of 12 h) with access to water and feed *ad libitum* for a period of 28 days. The experimental diets were prepared according to AIN-93G (Reeves et al., 1993) modified by the addition of crystalline cholesterol (0.5 g 100 g^{-1} diet) and sodium cholate (0.25 g 100 g^{-1} diet). The four groups were divided according to the amount of JSF added to the diet: 0 (control); 0.5, 1.5 and 3.0 g 100 g^{-1} diet (Table 1). Feed consumption and animal weight were monitored weekly, in order to calculate the average daily consumption (ADC), the average daily weight gain (ADG) and the feed efficiency ratio (FER). At the end of the experiment, the animals were fasted for about 12 h, and then were anesthetized with thiopental sodium, intraperitoneally.

Blood was removed from the heart and then centrifuged at 2,500 x *g* for 5 min for the collection of the plasma, which was stored at -20°C. The liver was removed by median laparotomy, washed with 0.9% saline solution, weighed and stored at -25°C for further analyses.

Blood analyses

The analyses were performed with blood plasma. For all tests, Lab

Table 1. Composition of the experimental diets.

Ingredient	Diet (g 100 g^{-1} diet)			
	Control (0)	0.5	1.5	3.0
Starch	40	40	40	40
Casein	20	20	20	20
Sucrose	10	10	10	10
Oil	10	10	10	10
Cellulose	5	5	5	5
Mineral mixture	3.5	3.5	3.5	3.5
Vitamin mixture	1	1	1	1
Methionine	0.5	0.5	0.5	0.5
Cholesterol	1	1	1	1
Sodium cholate	0.25	0.25	0.25	0.25
JSF[1]	0	0.5	1.5	3.0
Kaolin	8.75	8.25	7.25	5.75
Caloric value (cal g^{-1})	3,022.32	2,975.31	2,912.32	3,019.97

[1]JSF, Jabuticaba skin flour.

Test kits were used. Analyses of total cholesterol and of the HDL-c fraction were performed, as well as triacylglycerols, cholesterol in VLDL + LDL fractions, activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamyl transferase (GGT) were also determined.

Analyses in the liver

Moisture, lipid and total cholesterol

The livers were lyophilized until constant weight and finely ground. Moisture and lipid content were determined using the methods proposed by AOAC (2005). The extraction of cholesterol was carried out with isopropanol (Haug and Hostmark, 1987) and the dosage was performed in the same way for the blood analyses.

Thiobarbituric acid reactive substances

The peroxidation of lipids isolated from the liver of animals was determined by the formation of thiobarbituric acid reactive substances (TBARS), according to Winterbourn et al. (1981). The pigment produced by the colorimetric reaction was read in a spectrophotometer at 535 nm. The TBARS concentration was calculated from the standard curve of 1,1,3,3 tetraethoxypropane. The results were expressed as n moles of malondialdehyde acid (MDA) g^{-1} protein.

Histopathological analysis

A liver fragment from each animal was fixed in 10% formalin. The fragments were soaked in paraffin, sectioned (5 µm) and stained with hematoxylin and eosin (HE method). The slides were evaluated under a microscope and identified for the presence of hepatic steatosis, considering mild (+), moderate (+ +) or severe (+ + +) lesion.

Statistical analysis

The experimental design was completely randomized with four treatments, which were the control group (0%) and the groups containing 0.5, 1.5 and 3.0% JSF, with eight replicates, and each animal represented an experimental plot. For the analyses of ADC, ADG and FER, split plots in time were used. The software Sisvar (Ferreira, 2003) was used to perform the analysis of variance and, when significant, the regression analysis was performed, with $p \leq 0.05$.

Table 2. Proximate composition[1] of the jabuticaba skin flour (g 100 g^{-1}).

Constituent	Content
Moisture	21.69 ± 0.20
Lipids	1.59 ± 0.41
Crude protein (N × 6.25)	5.53 ± 0.18
Ash	5.46 ± 0.57
Insoluble fiber	27.51 ± 1.08
Soluble fiber	6.05 ± 0.78
Total dietary fiber	33.56 ± 1.45
NFE [2]	32.17 ± 1.30

[1]Data are the mean of triplicate ± standard deviation. [2]NFE, nitrogen-free extract.

RESULTS AND DISCUSSION

The proximate composition of JSF is presented in Table 2. The lipid content was low, but higher than that found by Leite et al. (2011) and Lima et al. (Lima 2011b) in lyophilized jabuticaba skin samples (1.27 and 1.16 g 100 g^{-1} DM, respectively). Lima et al. (2011a) reported contents of crude protein and ash (1.16 and 4.40 g 100 g^{-1} DM, respectively) for Sabará skins, lower than those found in this study. On the other hand, the contents of soluble (7.73 g 100 g^{-1} DM) and insoluble fiber (35.13 g

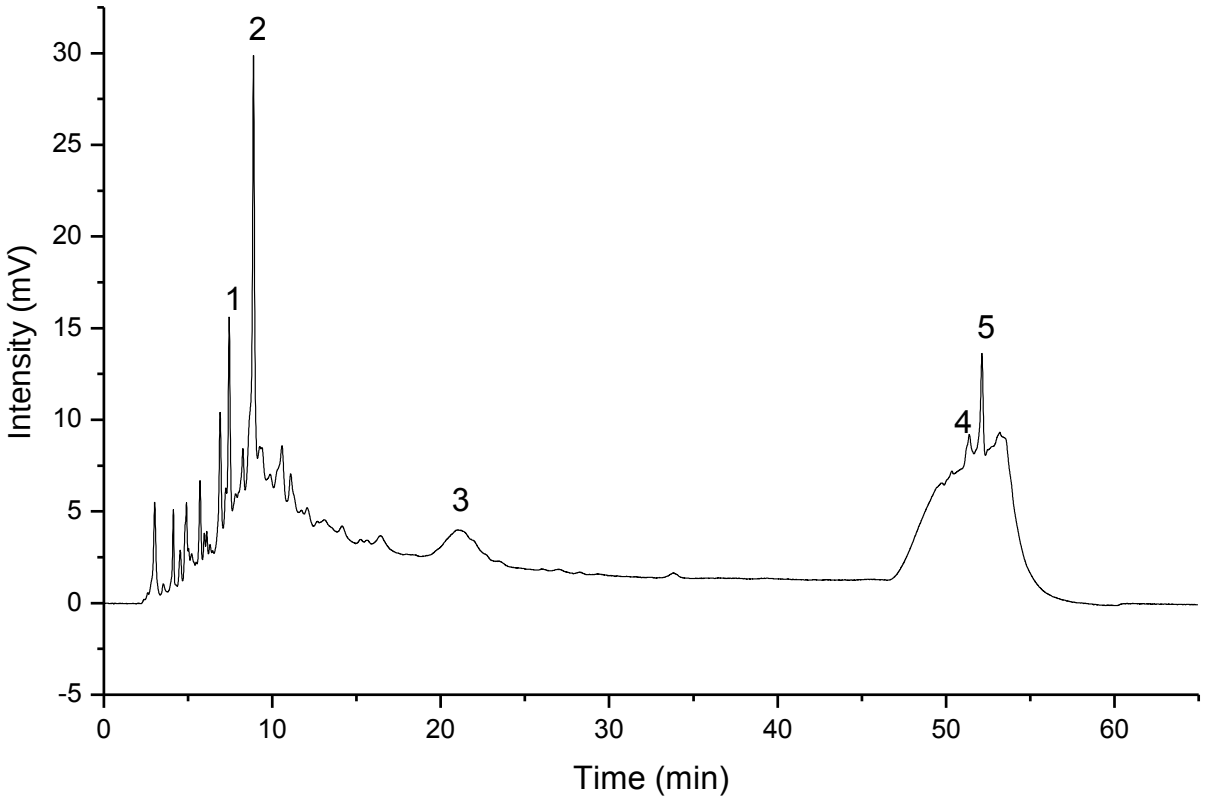

Figure 1. Chromatogram of the JSF extract peak identification: 1- gallic acid, 2- gallocatechin, 3- epicatechin, 4- ellagic acid, 5- salicylic acid.

Table 3. Phenolics average in jabuticaba skin flour, by HPLC.

Phenolic compound	Phenolic content (g 100 g^{-1} DM)
Gallic acid	0.11
Gallocatechin	0.01
Epicatechin	1.9 ±
Ellagic acid	0.12
Salicylic acid	0.54
Total	2.68

100 g^{-1} DM) were higher than those reported by Lima et al. (2011a) (6.80 g 100 g^{-1} DM for soluble fiber and 26.43 g 100 g^{-1} DM for insoluble fiber). The component with the highest content was dietary fiber. These differences are probably inherent in harvest, among other factors. In the chromatographic analyses of the JSF extract, phenolic acids and flavonoids were identified, presenting the following quantitative order: epicatechin > salicylic acid > ellagic acid > gallic acid > gallocatechin (Figure 1) and the total content of phenolic compounds was 2.68 g 100 g^{-1} DM (Table 3). The anthocyanins cyanidin-3-glucoside and delphinidin-3-glucoside were identified in Sabará JSF by HPLC (Leite-Legatti et al., 2012). JSF has proven antioxidant action (Leite-Legatti et al., 2012), probably due to the high content of phenolic compounds. In Table 4, the analyses of ADC, ADG and FER are shown. The analyses of variance for these variables using the split-plot scheme in time showed significant difference at 1% by f test, just for the time. Lenquiste et al. (2012) evaluated the effect of lyophilized jabuticaba skin on rats, in the proportions 0, 1, 2 and 4% added to the diets rich in fat, and did not observe significant statistical differences in the average daily consumption (ADC) and average daily weight gain (ADG) of the animals. These results suggest that the addition of JSF did not affect the palatability of the diets.

The analyses of total cholesterol, triacylglycerols and GGT activity carried in the blood of the animals showed no significant difference and averages are shown in Table 5. The diet supplemented with 3.0% JSF had the highest increase in the level of HDL-c, compared to the control (Figure 2), that was, 20.23%. In a research conducted by Lenquiste et al. (2012), using lyophilized jabuticaba skin, the authors also reported that there was no significant difference for the levels of total cholesterol and triacylglycerols between the diets supplemented with jabuticaba skins and the control and that there were statistical differences for the level of HDL-c. The diets supplemented with 2 and 4% jabuticaba skins showed the lowest values of HDL-c. Phenolic compounds, which

Table 4. Average daily consumption (ADC), average daily weight gain (ADG) and feed efficiency ratio (FER) of animal during the experimental phase.

Parameter	Diet			
	Control (0% JSF[1])	0.5% JSF	1.5% JSF	3.0% JSF
ADC (g)	17.35	17.11	18.55	17.94
ADG (g)	1.43	1.15	1.46	1.43
FER	0.09	0.07	0.08	0.09

[1]JSF, Jabuticaba skin flour.

Table 5. Total cholesterol, triacylglycerols (TAG) and gama glutamyl transferase (GGT) of animal during the experimental phase.

Parameter	Diet			
	Control (0% JSF[1])	0.5% JSF	1.5% JSF	3.0% JSF
Total cholesterol	193,14	165,99	147,56	166,37
TAG	29.72	25,3	26,73	26,10
GGT	4,87	4,46	3,72	3,82

[1]JSF, Jabuticaba skin flour.

are antioxidants, may be responsible for the increase in HDL-c. The accumulation of cholesterol in erythrocytes, leukocytes, platelets and endothelial cells can lead to a reduction in the antioxidant defense systems and cause an increase in the concentration of reactive species (Afonso et al., 2013). Flavonoids act to inactivate free radicals in hydrophilic and lipophilic cellular compartments and have the ability to donate hydrogen atoms, inhibiting chain reactions caused by free radicals (Degáspari and Waszczynscyj, 2004). The flavonoids identified in JSF may have acted as lipophilic antioxidants, attenuating the oxidative stress associated with cardiovascular diseases, which may have increased HDL levels.

One possible mechanism proposed for the reduction of plasma cholesterol levels is the formation of insoluble complexes with bile acids, increasing their fecal excretion, therefore there is no reabsorption of bile acids; cholesterol is then used to synthesize new bile acids, thus decreasing the level of cholesterol (Mäkynena et al., 2013). Studies indicate that phenolic compounds can induce an increase in the fecal excretion of bile acids (Lee et al., 2010). The high content of these compounds in JSF may have increased the fecal excretion of sterols, bile acids and non-fecal cholesterol, contributing to the occurrence of the antihyperlipidemic action of the flour. Although, not significant, a decrease in total cholesterol was observed in the animals. Analyses of the enzymes AST and ALT are used to identify changes in the function of the liver and of the biliary tract, and the enzyme GGT identifies biliary lesions. There was no statistical difference between the control group and the treatments in relation to the enzyme GGT (Table 5). It was observed in Figure 2 that the groups that received JSF showed activities of AST and ALT significantly lower than the control group ($p \leq 0.05$). JSF is probably acting as a hepatoprotective, since all groups received 1.0 g cholesterol, therefore with an accumulation of fat in the liver. The results suggest that this accumulation of fat was less harmful to the animals that received the treatment with JSF.

Total flavonoids extracted from the fruit *Rosa laevigata* Michx showed a significant hepatoprotective effect on mice that suffered liver damage, caused by the ingestion of paracetamol. The results were based on the determination of liver enzymes and histopathological tests (Liu et al., 2001). The authors suggest that this effect is due to the antioxidant potential of flavonoids. The decrease in the levels of AST and ALT, caused by the addition of JSF in the diets, may be due to its high content of phenolic compounds. Confirming these results, the histopathological study revealed a significant decrease in macrovesicular steatosis in the liver of the animals fed the diet containing 3.0% JSF (Figure 3). The increase in cholesterol intake has been associated with lipid peroxidation processes. Analysis of the lipid peroxidation index in the plasma of hamsters treated with hypercholesterolemic diets show an increase in the occurrence of thiobarbituric acid reactive substances (TBARS) (Sánchez-Muniz, 2012). Diets containing 1.5 and 3.0% JSF reduced the production of TBARS by about 50% in the liver of animals with an average weight of 140 g, indicating that JSF conferred protection against oxidative attack (Figure 1). Lenquiste et al. (2012) analyzed the antioxidant potential, by the 2,2′-azinobis-(3-etilbenzotiazolin-6-sulphonic) acid (ABTS) method, of the

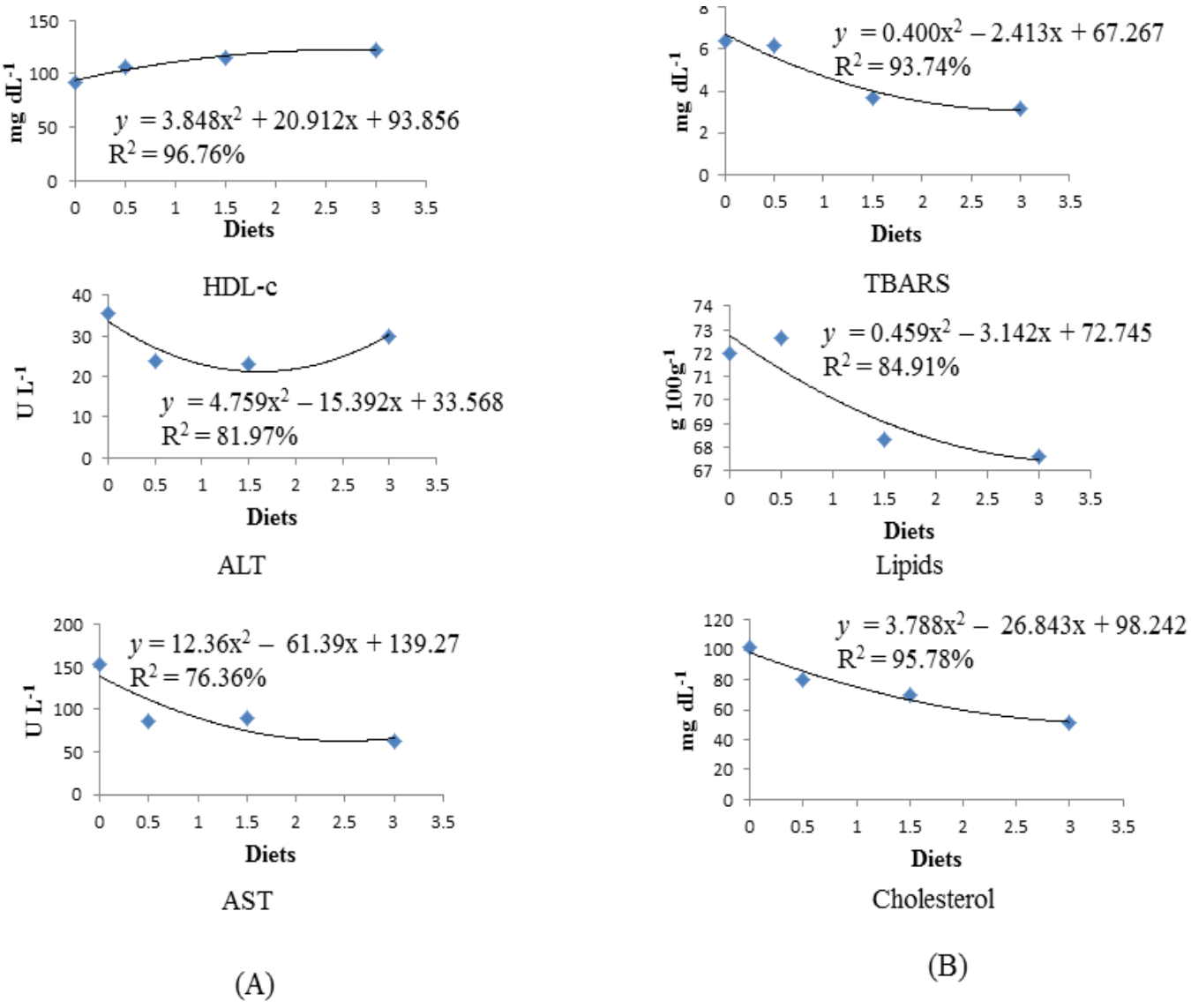

Figure 2. Analyses in the blood (A) and liver (B) of animals, after four weeks with the experimental diets ($p \leq 0.05$).

plasma of rats treated with a diet containing 0, 1, 2 and 4% lyophilized jabuticaba skin, and observed that there was an increase in the levels of ABTS with the diet with up to 2% jabuticaba skin for animals with an average weight of 250 g, also protecting against oxidative attack. The colonic microbiota causes the fermentation of phenolic compounds that occur in matrices rich in fibers, and releases absorbable compounds (Liu et al., 2001).

Regarding flavonoids, colonic bacteria share the heterocyclic ring and degrade flavonoids into phenyl acids that can be absorbed. After absorption, they are conjugated in the liver by glucuronidation, sulfation, methylation, or are metabolized into smaller phenolic compounds. They are able to inhibit cell proliferation and oxidative stress, as well as induce enzyme detoxification, apoptosis and activate the immune system (Guida-Cardoso et al., 2004). The phenolic acids and flavonoids present in JSF, after being metabolized and deposited in the liver of the animals, may be acting to inhibit oxidative stress and cause body detoxification. There was no significant difference for liver moisture, and the average moisture content was 52.79 g 100 g^{-1}. There was a reduction in the level of cholesterol and lipids in the liver of the animals, compared to the control. The high content of fiber and phenolic compounds in JSF may be responsible for these results. Hepatic cholesterol is the result of the balance between the cholesterol acquired through food, the cholesterol synthesized by the body and the cholesterol eliminated by the liver. Cholesterol has a ring structure, which the human body is unable to metabolize into CO_2 and H_2O, and is then eliminated by the liver as unchanged cholesterol into the bile, which

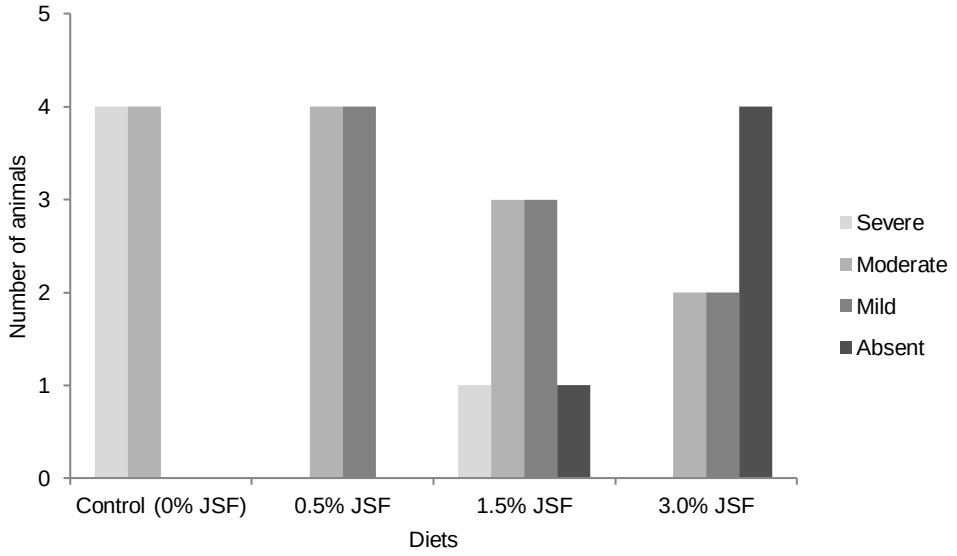

Figure 3. Distribution of macrovesicular steatosis observed in the histopathological analysis of the liver of the animals after four weeks of experiment. JSF, Jabuticaba skin flour.

transports it to the intestine for elimination, either as a component of plasma lipoproteins, or as bile salts, which are excreted in the feaces (Pérez-Jimenéz, 2009).
The animals fed diets supplemented with 3.0% JSF showed a decrease in the cholesterol level of 37.39% in the liver, when compared to the animals fed the control diet (Figure 1). The phenolic compounds in JSF may be acting to cause the decrease in cholesterol synthesis.

Conclusion

The JSF was effective in the protection against dyslipidemia, because it increases the serum level of cholesterol in HDL. The JSF has a high content of phenolic compounds and these components may be responsible for the occurrence of the hepatoprotective effect observed. Furthermore, the JSF has an antioxidant activity, since it protected the liver against lipoperoxidation. The phenolic acids and flavonoids identified epicatechin, salicylic acid, ellagic acid, gallic acid and gallocatechin, probably are responsible for this antioxidant activity.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Antitrichomonal activity of *Acanthospermum hispidum* D. C. (Asteraceae)

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Acanthospermum hispidum **(Asteraceae), used ethnomedically in the treatment of inflammatory conditions and fever, was evaluated for antiprotozoal activities such as trypanocidal and antiplasmodial effects. This study was carried out to investigate the anti-trichomonal potential of the plant. The air-dried leaf was extracted successively with petroleum ether, chloroform, ethylacetate and methanol using the soxhlet extraction method. Bioactivity-guided fractionation of the most active extract was carried out using the vacuum liquid chromatographic technique for antitrichomonal activity using** ***Trichomonas gallinae in vitro.*** **The ethyl acetate extract (A3) was the most active extract with LC50-LC90 values of 0.58-1.06 and 0.58-1.05 mg/ml at 24 and 48 h, respectively. Subfraction C7 had the highest antitrichomonal activity with 0.25-0.66 and 0.25-0.54 mg/ml at 24 and 48 h, respectively comparable to the activity of metronidazole at 0.20-0.39 and 0.16-0.36 mg/ml at 24 and 48 h, respectively.** ***A. hispidum*** **possessed antitrichomonal activity which resided in the chloroform portion of the ethyl acetate extract of the plant.**

Key words: *Trichomonas gallinae*, vacuum liquid chromatography, antiprotozoal.

INTRODUCTION

Diseases such as trypanosomiasis and trichomoniasis are taking their toll in terms of mortality and morbidity on human and animal populations in the developing countries. Available data showed that the annual incidence of trichomoniasis is more than 170 million cases worldwide (WHO, 1995). Trichomoniasis encompasses a broad range of symptoms ranging from a state of severe inflammation and irritation with a frothy malodorous discharge to a relatively asymptomatic carrier state (Swygard et al., 2004). The emergence of drug-resistant strains and dose-limiting toxic effects of existing drugs have complicated the treatment of parasitic protozoan diseases. Medicinal plants are a reservoir of bioactive compounds and therefore, effort is focused on them for potentially useful anti-infective agents. *Acanthospermum hispidum* DC (Asteraceae) is commonly called Bristly starburr, bristly tee or hispid starburr has its synonym as *A. humile.*

Acanthospermum is from the Greek words 'acantha' (thorn) and 'sperma' (seed) and refers to the prickly fruit while *hispidum* is Latin which means rough, bristly or prickly (David et al., 1989). Ethnomedicinally, *A. hispidum* is used in the treatment of yellow fever, malaria and stomach disorder (Denis, 2002; Mann et al., 2003). It is also used in some parts of South America as sudorific and diuretic. The plant has been scientifically investigated for its antibacterial and antiviral (Summerfield et al., 1997; Anani et al., 2000; Kamanzi et al., 2002; Fleischer et al., 2003; Hoffman et al., 2004), abortive and teratogenic (Lemonica and Alvarenga, 1994), antifeedant (Kraus et al., 1994; Rai and Achanya, 1999), antimalarial (Sanon et al., 2003; Gafon et al., 2012), immunostimulatory

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(Summerfield and Sallmuller, 1998), antitrypanosomal, antileishmania (Kamanzi et al., 2004; Ganfon et al., 2012) activities.

The plant has been reported to possess sesquiterpene lactones such as acanthospermal B, acanthospermal B epoxide, hispidunolide A and B (Herz and Kalyanaraman, 1975; Jakupovic et al., 1986; Kraus et al., 1994; Cartagena et al., 2000; Arena et al., 2011). In addition, glycosides and flavonoids have been reported to be present in the aerial part of the plant (Nair et al., 1976; Edewor and Olajire, 2011). *A. hispidum* has been shown to be a potentially useful plant in the treatment of protozoan infections; though, the antitrichomonal activity had not been reported. This study was carried out to investigate the activity of various extracts and fractions of *A. hispidum* on a protozoan parasite, *Trichomonas gallinae*.

MATERIALS AND METHODS

Drugs, reagents and solvents

Metronidazole tablet (May and Baker, Nigeria; Batch No. IU 268), methanol, ethyl acetate, chloroform, petroleum ether (BDH, UK), dimethylsulphoxide, sodium chloride, potassium chloride, calcium chloride, glucose, sodium hydrogen phosphate (BDH), sodium hydrogen bicarbonate (East Anglia Chemicals, UK) and sulphuric acid (Scharlau, Spain).

Plant collection and preparation

A. hispidum D. C. (Asteraceae) was collected at Ile-Ife, Nigeria in July. Plant authentication was done by Dr. H. C. Illoh of the Botany Department, Obafemi Awolowo University and compared with herbarium specimen IFE 5986. The leaf was oven-dried at 40°C, powdered using the grinding machine (Christy Norris) and stored appropriately in an amber-coloured bottle until required.

Plant extraction

The powdered leaf of *A. hispidum* (2.0 kg) was successively extracted with petroleum ether, chloroform, ethyl acetate and methanol for 48 h each using the soxhlet extraction method. The extracts were concentrated *in vacuo* at 35°C to give petroleum ether (A_1), chloroform (A_2), ethyl acetate (A_3) and methanol (A_4) extracts. The yields obtained were 0.95, 2.63, 1.00 and 1.27%, respectively.

Vacuum liquid chromatography (VLC)

The ethyl acetate extract (A_3, 18 g) was subjected to vacuum liquid chromatography (VLC) using silica (Burgoyne, India) and eluted with gradient solvents of petroleum ether/chloroform, 100% chloroform, chloroform:methanol (1:1) to give sub fractions B_{1-7}. Further fractionation of B_2 (2.7 g) by VLC using gradient solvent systems of petroleum ether and chloroform, yielded purified fractions C_{1-8}.

Thin layer chromatography analysis of A_3 and its fractions

Extracts and fractions were monitored by thin layer chromatography (TLC) on precoated silica gel 60 F_{254} plates (Merck®) eluted with the following solvent systems: I) petroleum ether: chloroform (1:1), II) petroleum spirit:chloroform (1:9), III) chloroform:ethyl acetate (4:1), IV) chloroform:methanol (3:2) and V) ethyl acetate:methanol (3:2). The chromatograms were examined under the UV light at 254 and 366 nm then sprayed with 10% H_2SO_4.

Preparation of metronidazole (positive control), extracts/fraction

Metronidazole, extract/fraction (4 mg) was dissolved in 0.25 ml dimethyl sulfoxide (DMSO) and made up to 1 ml solution using the Locke-egg (LE) medium to give 4000 µg/ml. Serial dilution was done to obtain 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.625 and 7.8125 µg/ml. The LE medium was prepared by thoroughly mixing 50 ml Ringer's solution, 1 ml bovine serum and 1 ml 10% glucose solution.

Parasite

T. gallinae parasites were isolated from the mouth and upper crop of *Columba livia* (local pigeon) using sterile cotton-tipped swab sticks immersed in physiological saline solution (Narcisi and Secor, 1996). The parasites were cultured in egg slant tubes suspended in LE medium and incubated vertically at 37°C until ready for use (Omisore et al., 2005).

Antitrichomonal bioassay

For each extract/fraction, 50 µl of 10^4 organisms/ml of *T. gallinae* parasites was added to 150 µl of test extract/fraction in a sterile 96-microwell flat bottom plate (Nunc) with metronidazole and DMSO-LE medium as positive and negative controls, respectively. The plates were incubated at 37°C. At 24 and 48 h, surviving (motile) parasites were counted per ml with the aid of a microscope. At least each concentration was done in triplicate analyses.

Data and statistical analysis

The percentage mortality of the parasites was calculated as 100 × [100-(*A*/*B*)], where *A* is the number of motile organisms in the test groups and *B* is the number of motile organisms in the negative control group. The LC_{50} and LC_{90} values were derived from the respective percentage mortality values using Microsoft Excel (2007) and subjected to statistical analysis using the one-way analysis of variance (ANOVA) followed by the post-hoc Dunnett test (Graphpad Instat, 2003).

RESULTS

The results are presented in Tables 1 to 3. The LC_{50} and LC_{90} values of A_1, A_2 and A_3 reduced non-significantly ($P>0.05$) over time from 24 to 48 h. The activities of A_1, A_2 and A_3 were comparable to the positive control, metronidazole; however, A_4 was significantly different [F

Table 1. Antitrichomonal activity of the extracts of *Acanthospermum hispidum* DC. (Asteraceae) using *Trichomonas gallinae*.

Extract	24 h		48 h	
	LC_{50} (mg/ml)	LC_{90} (mg/ml)	LC_{50} (mg/ml)	LC_{90} (mg/ml)
A_1	0.81±0.23	1.44±0.30*	0.61±0.02	1.16±0.08*
A_2	0.63±0.05	1.23±0.13*	0.60±0.02	1.15±0.08*
A_3	0.58±0.02	1.06±0.05*	0.58±0.00	1.05±0.00*
A_4	1.07±0.27*	1.66±0.54*	0.89±0.04*	1.06±0.17*
Metronidazole	0.20±0.02	0.39±0.01	0.16±0.01	0.36±0.02

LC_{50}, $LC_{90:\ values}$ are mean ± standard error of the mean (SEM). *Significantly different from metronidazole. A_1, Petroleum spirit extract; A_2, chloroform extract; A_3, ethyl acetate extract; A_4- methanol extract.

Table 2. Antitrichomonal activity of the fractions of the ethyl acetate extract (A_3) of *A. hispidum* at 24 and 48 h.

Extract	24 h		48 h	
	LC_{50} (mg/ml)	LC_{90} (mg/ml)	LC_{50} (mg/ml)	LC_{90} (mg/ml)
A_3	0.58±0.02	1.06±0.05	0.58±0.00	1.05±0.00
B_1	1.13±0.15	2.00±0.27	0.70±0.10	1.44±0.17
B_2	1.15±0.15	1.97±0.21	0.99±0.14*	1.77±0.11*
B_3	1.09±0.25	2.03±0.39	0.79±0.15	1.54±0.18*
B_4	1.54±0.46*	2.70±0.86	1.36±0.37*†	2.43±0.58*†
B_5	1.04±0.13	1.95±0.27	0.77±0.04	1.42±0.09*
B_6	1.06±0.13	2.05±0.25	0.85±0.15	1.60±0.10*
B_7	1.87±0.57*†	3.11±1.11*	0.99±0.23*	1.86±0.29*
Metronidazole	0.20±0.02	0.39±0.01	0.16±0.01	0.36±0.02

LC_{50}, $LC_{90:}$ values are mean ± standard error of the mean (SEM). *Significantly different from metronidazole. †Significantly different from A_3.

(4, 10) 3.96, P = 0.035] from metronidazole at 24 h (Table 1). A_3 gave the highest activity as it had the lowest LC_{50} and LC_{90} values at 24 and 48 h. Thus, A_3 (22 g) was fractionated using VLC and sixteen eluates were obtained which were bulked into 7 fractions (B_{1-7}) according to their TLC profiles. At 24 h, all the fractions except B_4 and B_7 had similar activity compared with metronidazole while only the LC_{50} of B_7 was significantly different [F(8, 18) 3.099, P = 0.022] at 24 h from the mother extract, A_3 (Table 2). At 48 h, the LC_{50} values of B_2, B_4 and B_7 were significantly different from metronidazole while only B_4 was different from A_3 [F (8, 18) = 3.624, P = 0.011). All the fractions except B_1 were significantly different from metronidazole when their LC_{90} values were compared. Although, B_{1-3} and B_{5-6} had comparable activities, B_2 (2.9 g), which had the highest weight, was further purified. Eight bulked fractions were obtained (C_{1-8}) according to their TLC characteristics.

At 24 h, all the sub-fractions except C_5, C_6 and C_7 were significantly different [F (9, 20) = 5.52, P = 0.0007] from metronidazole while at 48 h, only C_6 and C_7 were comparable [F (9, 20) 41.46, P<0.0001] to metronidazole. However, they were significantly different [F (9, 20) 34.29, P<0.0001) from A_3 (Table 3). Thus, the activities of the two sub-fractions were significantly comparable to metronidazole at both time points and showed better activity than A3.

DISCUSSION

The extracts and fractions of *A. hispidum* gave moderate to remarkable levels of mortality when tested on the protozoa, *T. gallinae*. The activity of A1, A2 and A3 implies the bioactive component may be non-polar while the polar methanolic extract exhibited minimal activity when compared with metronidazole. It thus, appeared that the bioactive component (s) is/are relatively apolar. In addition, it seemed the extracts exhibited biostatic action on the protozoa which was not sustained after 24

Table 3. Antitrichomonal activity of subfractions of B_2 using *Trichomonas gallinae* at 24 and 48 h.

Extract/sub-fraction	24 h		48 h	
	LC_{50} (mg/ml)	LC_{90} (mg/ml)	LC_{50} (mg/ml)	LC_{90} (mg/ml)
A_3	0.58±0.02	1.06±0.05	0.58±0.00*	1.05±0.00*
C_1	1.00±0.08*	1.70±0.09*†	0.73±0.06*†	1.40±0.09*†
C_2	1.10±0.12*	1.80±0.10*†	0.67±0.08*	1.25±0.09*
C_3	0.74±0.38*	1.44±0.03*†	0.68±0.05*	1.29±0.06*
C_4	0.94±0.16*	1.73±0.20*†	0.85±0.04*†	1.49±0.13*†
C_5	0.62±0.00	1.21±0.01*	0.59±0.01*	1.14±0.04*
C_6	0.26± 0.03	0.60±0.05†	0.28 ± 0.01†	0.52± 0.01†
C_7	0.25 ± 0.01	0.66± 0.01†	0.25± 0.01†	0.54± 0.01†
C_8	0.81±0.04*	1.56±0.01*†	0.62±0.03*	1.18±0.10*
Metronidazole	0.20±0.02	0.39±0.01	0.16±0.01	0.36±0.02

LC_{50}, LC_{90}: values are mean ± standard error of the mean (SEM). *Significantly different from metronidazole. †Significantly different from A_3.

h. The increasing resistance to metronidazole in the treatment of trichomoniasis, with the various adverse effects observed in the use of the drug, has led to the search for bioactive agents in medicinal plants with potential antitrichomonal activity. Trichomonad species readily obtained for laboratory study are *T. muris* in mice and rats and *T. gallinae* from crop of pigeons (Smyth, 1996). *T. gallinae* was used because of its availability and morphological similarity to T. vaginalis *T. vaginalis*, the causative parasite for human trichomoniasis. Since the duration of survival and growth rate is inversely proportional to inoculum density, trichomonads can sometimes overgrow the media and die off within 36 to 48 h, thus the choice of the time points.

The most active subfractions are from the chloroform portion of the ethyl acetate extract. Deepa et al. (2004) reported that the ethyl acetate extract of *A. hispidum* possessed antibacterial and antifungal activities comparable to ciprofloxacin and clotrimazole, respectively. Non-polar compounds have been found effective as potential agents in the treatment of trichomoniasis. The pentacyclic triterpenoid, hederagenin, was reported as the antitrichomonal component of *Cussonia holtsi* (Araliaceae) with an IC_{50} of 2.8 µM (He et al., 2003). In addition, bartericins A and B as well as isobavachalcone (isolated from *Dorstenia barteri*) were reportedly active at 0.121 to 31.25 µg/ml, against *T. gallinarum* (Omisore et al., 2005). The antibacterial and antimalarial activities of *A. hispidum* have been ascribed to Acanthospermal B and other sesquiterpene lactones (Arena et al., 2011; Ganfon et al., 2012). It is therefore possible that the putative antitrichomonal constituent of *A. hispidum* belongs to the class of sesquiterpene lactones which abound in the plant.

Sesquiterpene lactones (SQLs) have been reported from 10 families of flowering plants; with the greatest numbers derived from the Asteraceae. The α-methylene γ-lactone moiety of this group of compounds is very reactive with the thiol groups of important biological components such as enzymes which make SQLs have diverse biological activity. Further studies on the most active subfractions may reveal a more active compound than the standard drug, metronidazole.

Conclusion

Bioactivity-directed purification of the leaf of *A. hispidum* using anti-trichomonal assay yielded subfractions C_6 and C_7 which had activity comparable to metronidazole, the positive control and had better activity than the mother ethyl acetate extract. The study further showed the potential usefulness of *A. hispidum* in treating protozoal infections.

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Full Length Research Paper

In vitro microrhizome production in *Decalepis hamiltonii*

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Microrhizomes were produced from leaf disc derived callus of a threatened endemic medicinal plant - *Decalepis hamiltonii.* Murashige and Skoog (MS) medium supplemented with 2 µM 6-benzyleaminopurine (BAP) and 6 µM 1-napthaleneacetic (NAA) acid was found to be optimum for rapid callus induction and establishment from leaf disc explants. Further differentiation of callus into microrhizome was conquered in MS medium supplemented with 4 µM indole-3-butyric acid (IBA) and 8 µM NAA. A maximum of 20 microrhizomes in a cluster was produced within 90 days. Yeast extract (0.05%) and polyvinylpyrrolidone (0.05%) further enhanced the microrhizome formation when supplemented along with plant growth regulators (PGRs).

Key words: *Decalepis hamiltonii*, microrhizome, herbal medicine, secondary metabolites.

INTRODUCTION

In recent years, there has been a gradual revival of interest in the use of medicinal plants in developed as well as developing countries since herbal medicines have been reported to be safe without side effects (Bahadur et al., 2007). Consequently, several medicinal plants becoming rare, endangered and threatened (RET) categories. *Decalepis hamiltonii* Wight and Arn. (Asclepiedaceae) is a glabrous extensively climbing shrub growing in moist deciduous forests, scrub jungles of Deccan peninsula and the Western Ghats of India. This is an endemic and endangered medicinal plant of southern peninsula. It prefers to grow along rocky slopes, big rock boulders and rocky crevices and small mounds at an altitude from 300 to 1200 m (Ravikumar and Ved, 2000). Rhizome extract of this plant is reported to have antioxidant, antibacterial, hepatoprotective, antiinflammatory, insecticidal, antidiabetic, anti-atherosclerotic, immunomodulatory and antitumour potentials due to the presence of bioactive principles such as decalepin, salicylaldehyde, vanillin anisaldehyde, 2-hydroxy 4-methoxy benzaldehyde and 5,7,4-trihydroxy flavanone 4'-o-β-D-glucoside (Srivastava and Shivanandappa, 2006, 2010; Harish and Shivanandappa, 2011; Ashalatha et al., 2010; Naveen and Khanum, 2010; Sumalatha et al., 2010; Thangavel et al., 2011). In natural habitat, this species is under severe threat due to poor seed setting and ruthless harvesting by traditional medical practitioners (Ravikumar and Ved, 2000). Among

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Abbreviations: MS, Murashige and Skoog medium; **NAA,** 1-napthaleneacetic; **BAP,** 6-benzyleaminopurine; **IBA,** indole-3-butyric acid.

different forms of plant cell and tissue culture systems, specific organ cultures such as root, rhizome and tubers have been reported to hold tremendous potential for *in vitro* production of uniform, high quality plant based medicines (Tripathi and Tripathi, 2003; Subathra et al., 2006; Majumdar et al., 2010; Poojadevi et al., 2013.

Micro-rhizomes have been induced in *Curcuma aromatica*, *Curcuma longa* and the efficiency of microrhizome formation was found to be determined by the concentration of cytokinins, sucrose and coconut milk (Naik and Naik, 2006). Production of microrhizomes *in vitro* has many advantages compared to plantlet production. Besides, the use for extraction of secondary compounds, microrhizomes are feasible to facilitate germ plasm exchange across national borders. Hence, a rapid reproducible protocol for microrhizome production from leaf disc derived callus of *D. hamiltonii* have been devised in this present study.

MATERIALS AND METHODS

Procurement of plant material

Young saplings of *D. hamiltonii* was collected from Sirumalai hills, Dindigul district, Tamil Nadu, potted and maintained in the department (SPKCES, M. S. University) green house for routine culture work. Identity of the plant was authenticated by Dr. K. Ravikumar, Assistant Director, Repository for Medicinal Plant Resources, FRLHT, Bangalore. Voucher specimen was deposited in departmental herbarium (F.No:144: SPKCESH). Chemicals and plant growth regulators (PGRs) used in this present study were procured from Himedia Laboratories, Mumbai, India.

Preparation of culture medium

Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) was used throughout the study. The basal medium was modified by adding plant growth regulators (PGRs) such as 1-naphthaleneacetic acid, indole-3-butyric acid and 6-benzylaminopurine (2 to 10 µM), 1 to 3% (w/v) sucrose, media adjuvants such as coconut milk [5 to 15% (v/v)], yeast extract [0.05% (w/v)] and polyvinylpyrrolidone [0.05% (w/v)]. pH of the medium was adjusted to 5.6 to 5.8. After adding 0.8 to 1.2% (w/v) agar, medium was melted in a microwave oven and dispensed in culture vials, then sterilized in an autoclave at 15 Psi and 121°C, for 15 min.

Surface sterilization of explants

Fresh leaves of *D. hamiltonii* were collected, thoroughly washed in running tap water and 0.5% (v/v) sodium hypochlorite (NaOCl) with a few drops of “Teepol” for 5 min. This was followed by a thorough wash in running tap water. Then leaves were transferred to sterile laminar air flow cabinet and surface sterilized in 0.05% (w/v) mercuric chloride ($HgCl_2$) solution for 5 min and rinsed thoroughly in sterile distilled water.

Callus establishment and microrhizome initiation

Surface sterilized leaves were trimmed into optimum size (0.5 to 1.0 cm). Two discs were aseptically placed per culture vial with sterile MS medium. Culture vials were incubated under complete darkness at 25 ± 2°C. Proliferated callus was sub-cultured in 10 to 15 days interval for further establishment. After establishment, 0.5 g of callus was transferred to liquid MS medium (50 ml) supplemented with indole-3-butyric acid (IBA) and 1-napthaleneacetic (NAA: 2 to 10 µM), coconut milk [5 to 15% (v/v)] and yeast extract [0.05% (w/v)]. Suspension cultures were incubated at 24°C in orbital shaker at 100 rpm. Callus was sub-cultured in 15 days interval by filtering the callus mass with several layers of sterile cheese cloth. Partially differentiated microrhizomes were sub-cultured into solid medium for further development. 10 replicates were maintained for each media combination, fresh weight of callus was measured 20 days after sub culture. Callus induction efficiency of PGRs in different concentration and combination were evaluated through one way ANOVA. All culture sets were maintained with 10 replicates. Mean values were calculated (Table 1) and interpreted with control. Significance was taken at the level of $P< 0.01$.

RESULTS

Among different PGR combinations used, MS medium supplemented with NAA (6 µM) along with BAP (2 µM) exhibited callus proliferation at the maximum level (2.9 ± 0.64 g) after 20 days from sub-culture (Table 1), (Figure 1c). Leaf explants often leached phenolic substances that inhibited the callus growth considerably, but this was suppressed by the addition of 0.05% polyvinylpirrolidone (PVP).

Among various levels of sucrose and agar tested, 2 and 1.2% respectively were found to be ideal for rapid callus induction. After establishment, callus was transferred into microrhizome differentiation (liquid) medium, sub-cultured at 15 days interval. In liquid medium, differentiation and development of microrhizomes were observed in a slow rate (Figure 1d and 1e). Hence, the partially developed microrhizome clusters were transferred to solid medium where they grow faster and produced higher number of microrhizomes (Table 2) within 20 days (Figure 1f). Microrhizome differentiation potential was evoked by NAA in combination with IBA, YE and CM (Table 2). Culture medium supplemented with 8 µM NAA + 4 µM IBA + 0.05%YE and 10% CM exhibited maximum efficiency (average: 16 numbers) on microrhizome development and was considered very ideal for producing large number of microrhizomes. During initial stages, microrhizomes were white in color and after 15 days, they turned into pale brown color (Figure 1f).

DISCUSSION

Plant tissue culture techniques have been useful for conserving germplasm of rare and endangered species and considered as an alternative to conventional field gene banks to safeguard against pests and environmental stresses (Dodds, 1991). Tissue culture of medicinal plants holds tremendous potential for *in vitro* production and exploration of herbal therapeutics. In this

Table 1. Callus proliferation from leaf disc explants.

Medium composition					Callus induction efficiency	Initial weight of callus (g)	Weight (g) after 20 days[a] (10 replicates)	ANOVA[b]	
								P - value	F - value
MS					-	0.5	0.5	-	-
NAA (µM)	**2,4- D (µM)**	**S (%)**	**A (%)**	**PVP (%)**					
2.0	-	2.5	0.8	0.05	+	0.5	0.68±0.18	0.1042[NS]	2.927
4.0	-	2.5	0.8	0.05	+	0.5	0.72±0.16	0.0019**	13.198
6.0	-	2.5	1.0	0.05	+++	0.5	0.72±0.21	0.0019**	13.198
8.0	-	2.0	1.0	0.05	+		0.76±0.25	1.97E-06**	47.287
10.0	-	2.0	1.2	0.05	+	0.5	0.78±0.19	3.85E-06**	42.682
-	2.0	2.0	1.2	0.05	+	0.5	0.80±0.21	5.38E-07**	57.241
-	4.0	2.0	1.2	0.05	+	0.5	0.80±0.24	5.38E-07**	57.241
-	6.0	2.0	1.2	0.05	+ +	0.5	1.60±0.50	1.11E-20**	2443.606
-	8.0	2.0	1.2	0.05	+ +	0.5	1.70±0.53	1.37E-20**	2386.983
-	10.0	2.0	1.2	0.05	+ +	0.5	1.70±0.62	1.37E-20**	2386.893
NAA (µM)	**BAP (µM)**	**CM (%)**	**S (%)**	**A (%)**	**PVP (%)**				
2.0	2.0	10	2.0	1.2	0.05	+ +	0.5	1.78±0.59	3.34E-23**	4674.064
4.0	2.0	10	2.0	1.2	0.05	+ +	0.5	1.8±0.61	1.37E-20**	2387.344
6.0	2.0	10	2.0	1.2	0.05	+ + + +	0.5	2.9±0.64	8.98E-26**	9039.249
2,4-D (µM)	**BAP (µM)**	**CM (%)**	**S (%)**	**A (%)**	**PVP (%)**				
2.0	2.0	10	2.0	1.2	0.05	+ + +	0.5	2.0±0.58	2.9E-21**	2839.667
4.0	2.0	10	2.0	1.2	0.05	+ + +	0.5	2.1±0.62	1.49E-22**	3956.045
6.0	2.0	10	2.0	1.2	0.05	+ +	0.5	1.6±0.56	1.27E-19**	1860.319
6.0	2.0	10	2.0	1.2	0.05	+ +	0.5	1.55±0.58	7.07E-17**	912.910
6.0	2.0	10	2.0	1.2	0.05	+ +	0.5	1.52±0.72	1.79E-19**	1789.952

+ Delayed response with very minimum callus growth, + + delayed response with normal callus growth, + + + quick response with normal callus growth, + + + + quick response with maximum callus growth; NS, Not Significant; ** significant at P< 0.01; b - all values were compared with MS basal medium, a - all values are mean weight (20 days after sub-culture) of 10 replicates ± SD; CM, coconut milk; S, sucrose; A, agar; PVP, polyvinylpyrrolidone

this present study, a rapid, reproducible protocol has been devised for the development of microrhizome from leaf disc derived callus of *D. hamiltonii*. Plant growth regulators were amended at different concentrations and combinations. However, the specific combination of the medium resulted in different extent of callus induction and differentiation of microrhizome. With the stimulus of endogenous or by addition of exogenous

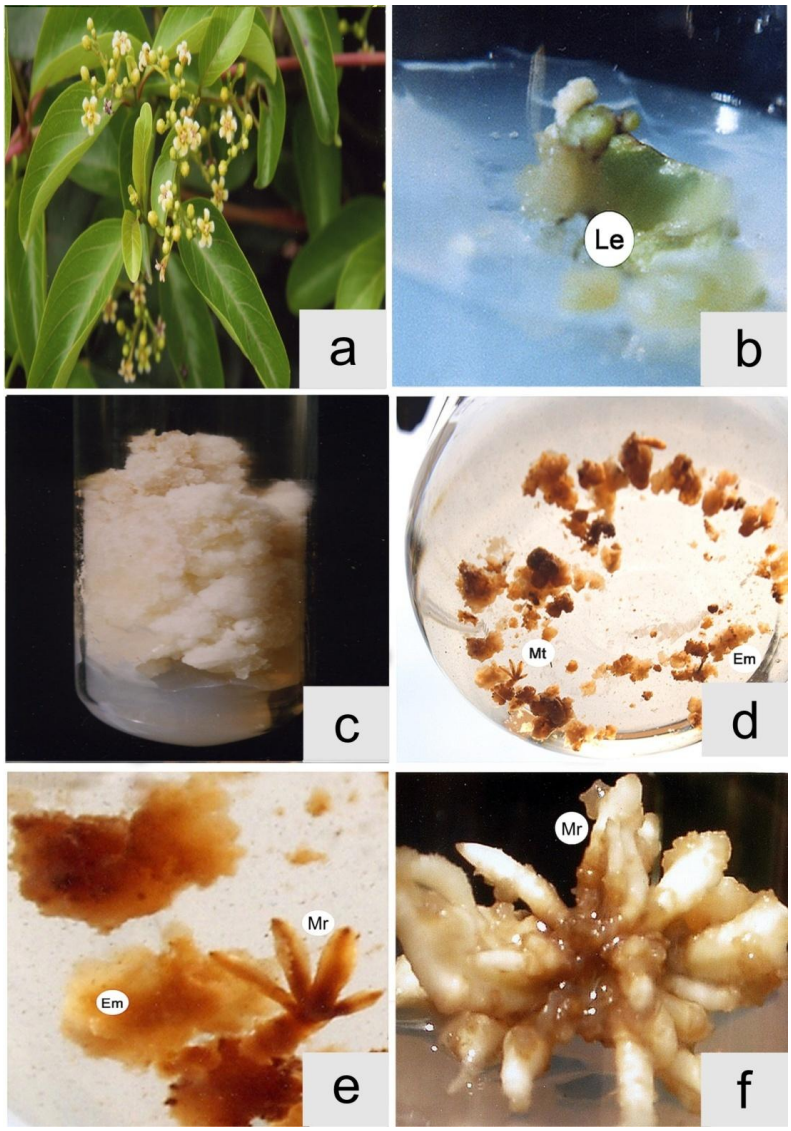

Figure 1. *In vitro* microrhizome development from leaf derived callus. **a.** *D. hamiltonii* - A twig with flower; **b.** leaf disc (Le) cultured on MS medium supplemented with 6 µM NAA + 2 µM BAP + 10% CM + 0.05% PVP. Callus proliferation after 10 days of inoculation; **c.** further establishment of friable callus (30 days after inoculation) - a stage suitable for liquid culture; **d.** friable callus after transfer into MS liquid medium supplemented with 8 µM NAA + 4 µM IBA + 10% CM + 0.05% PVP (developing microrhizome - Mr); **e.** further development of microrhizomes after 90 days; **f.** close up view of microrhizome cluster after transfer into solid medium.

growth regulators to the nutrient medium, cell division, and tissue differentiation could be induced. Callus obtained in this present study was friable in nature (Figure 1c).

Effect of PGRs on callus induction and establishment

Supplementation of auxins at suitable level is a generic requirement for any explant to induce callus. Callus was proliferated initially from the cut surfaces of leaf discs cultured in MS medium supplemented with NAA (Figure 1b). Within 15 days, well proliferated callus covered the entire explant.

Basal medium as well as BAP supplemented medium did not induce callus. In case of medium supplemented with BAP along with NAA callus, the proliferation was rapid. MS medium consist of 6 µM NAA, 2 µM BAP and 0.05% PVP promoted quick induction and rapid proliferation of callus compared to other combinations

Table 2. Microrhizome development from leaf disc derived callus.

Medium composition						Number of microrhizomes*
MS						-
NAA (µM)	**IBA (µM)**	**S (%)**	**YE (%)**	**CM (%)**	**A (%)**	
2.0	-	1.5	-	10	-	-
4.0	-	1.5	-	10	-	-
6.0	-	1.5	-	10	-	2 ± 0.13
8.0	-	1.5	-	10	-	5 ± 0.14
6.0	-	1.5	0.05	10	-	6 ± 0.17
8.0		1.5	0.05	10	1.2	7 ± 0.21
10.0	-	1.5	0.05	10	1.2	9 ± 0.24
8.0	2.0	1.5	0.05	10	1.2	12 ± 0.23
8.0	4.0	1.5	0.05	10	1.2	16 ± 0.26
8.0	6.0	1.5	0.05	10	1.2	11 ± 0.28

*All values are average of 10 replicates ± SD (after 90 days); YE, yeast extract; CM, coconut milk; S, sucrose; A, agar.

(Figure 1c). NAA could promote callus induction in several plants where 2,4-D or 2,4,5-T fails (Pawar et al., 2002). Callus induction and organogenesis was successful in several medicinal plants like *Petunia inflata*, *Withania somnifera*, *Commiphora wightii* and *Iphigenia indica* (Beek and Camper, 1991) from leaf disc explants with the supplementation of NAA in various concentrations.

In this present study, NAA in combination with BAP enhanced callus multiplication at the maximum level (Table 1) and this observation corroborates the previous reports (Giridhar et al., 2004). In *D. hamiltonii*, *in vitro* hairy root production was reported with low biomass (Giridhar et al., 2004; Sudha and Seeni, 2001). However, high efficiency of micro-rhizome production is achieved in this present study and it could be a better tool for large scale production of high value secondary metabolites available in the rhizome of this medicinally important plant.

Effect of modified sucrose and agar concentrations

Establishment of callus was successful only when the sucrose and agar concentrations were altered. In general, this plant stores high quantity of starch in all vegetative organs. Reducing the normal concentration of sucrose from 3 to 2% induced regenerative callus and the callus survived a longer time. Reduced level of sucrose in culture medium has been reported in medicinal plants like *Ficus lyrata* and *Solanum melongena* (Mukherjee et al., 1991).

Increasing the concentration of agar (1.0 to 1.2%) enhanced the survival and curtailed hyperhydricity based suppression during callus establishment. This could be attributed to the natural conditions where this plant is exposed to xeric habitats. Thus, creating a near natural environment facilitated growth of callus and differentiation of microrhizome significantly. Such a similar observation was made earlier in *Solanum surattense* by Pawar et al. (2002).

Effect of PGRs on microrhizome differentiation

Specialized organ cultures such as root, rhizome and tubers will provide an efficient means of biomass production due to fast growth and stable metabolite production. Callus mass was established and transformed into microrhizome in suspension and later on solid medium. During the course of such differentiation, profound influence of exogenous plant growth regulators was observed. As the bioactive principles of this plant are produced in rhizome, the *in vitro* rhizome production would be of much commercial interest and value. Microrhizome was differentiated at the maximum level in MS medium supplemented with IBA (4 µM), NAA (8 µM), yeast extract (0.05%) and coconut milk (10%).

This combination induced only four to seven microrhizomes in 90 days in suspension culture under agitated condition (Figure 1d). Rate of differentiation, further growth and development of microrhizome was successful only after transfer into solid medium containing similar hormone and adjuvant combinations (Figure 1f). Each cluster had a maximum of 20 microrhizomes and an average of 16 on solid medium. Callus mediated microrhizome was also produced from *Hemidesmus indicus* (Sreekumar et al., 1998), *Bunium persicum* (Grewal, 1996) and *Solanum tuberosum* (Piao et al., 2003) with various hormone supplementations. Microrhizomes have also been developed *in vitro* from various rhizomatous medicinal plants such as *Zingiber*

officinale (Sharma and Singh, 1995), *Curcuma aromatic* (Nayak, 2000) and *Curcuma longa* (Sunitibala et al., 2001). In all these reports, supplementation of PGRs such as NAA and IBA (2 to 15 µM), adjuvants like coconut milk, maleic hydrazide, activated charcoal and yeast extract were found to be crucial for microrhizome development. Photoperiod was reported to play a key role in induction of storage organs such as rhizome and tuber *in vitro* (Jean and Cappadocia, 1991). In the present study, cultures incubated in 16 h photoperiod produced more number of microrhizomes. Under 16 h light regime, large number of tubers have been produced in *Dioscorea abysinica* and *Dioscorea alata*, whereas continuous dark conditions hindered the tuberization process. Compared to other forms of cell aggregates, microtubers, microrhizomes and adventitious tuberous roots could serve as better alternative for large scale extraction of secondary metabolites (Jean and Cappadocia, 1991; Xie et al., 2000). Accordingly, the protocol devised in this present study will find an immense application in pharmaceutical industries for extraction and formulation of herbal products.

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