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Examples:

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

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

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Full Length Research Paper

'Omics' approaches in tomato aimed at identifying candidate genes for ascorbic acid accumulation in the fruit

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Tomato (*Solanum lycopersicum*) is one of the most important vegetables in the world with significant importance for human health and nutrition. This species has long served as model system for plant genetics, development, physiology, pathology, and fleshy fruit ripening, resulting in the accumulation of many genetic and genomic resources. In addition, the tremendous development of high-throughput technologies, such as transcriptomics, metabolomics and proteomics, collectively denoted as 'omics' technologies, has led to a huge collection of data and platforms today available on the net. Nowadays, identifying all the components of a single biological system is within our means; however, assigning function to genes, proteins and metabolites is still a daunting task. Major challenges include interpretation and integration of large datasets to understand the principles underlying the regulation of genes, metabolites and proteins, and how their combined interactions associate with variation in phenotype. In this review, we will focus on the role of the different high-throughput technologies in enhancing tomato breeding particularly for fruit quality traits. We also describe how two 'omics' approaches could be combined in order to identify candidate genes for the genetic control of ascorbic acid accumulation in tomato fruit. We report the example of transcriptomic and genomic approaches established on the use of different high-throughput platforms available for tomato.

Key words: Tomato, introgression lines, quality trait, genomics, transcriptomics, candidate gene, single nucleotide polymorphism (SNPs).

INTRODUCTION

Nowadays, by taking the advantages of development of new sequencing technologies, the genome sequences of several plant species have been revealed. Among the almost 30 plant genomes publicly available, there is the tomato (*Solanum lycopersicum*) genome, whose sequencing started at the end of 2004 in the framework of the International Solanaceae Genome Project (SOL) and completed at the end of the year 2011 (Tomato Genome Consortium, 2012). The tomato is one of the three most important vegetables in the world with significant importance for human health and nutrition. In the last years, its global production has increased approximately 10% since for many countries it is a significant source of vitamins and minerals (Giovannucci et al., 2002). Moreover, tomato has been always considered as model species for fleshy fruit development and ripening, as well as for genomics studies of other Solanaceae (Mueller et al., 2005). For these reasons many genetic and genomic resources have been

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developed for this species, including databases for transcriptomics, metabolomics and proteomics data, which are now available on the net (Fei et al., 2011). Indeed, in the post-genomic era, high-throughput technologies as microarray, mass-spectrometry and protein chip, have led to the collection of a large amount of data developed by the scientific community. These techniques allow measuring thousands of variables (genes, metabolites, proteins) simultaneously across populations.

The data generated by these techniques are often collectively denoted as 'omics' data (Joyce and Palsson, 2006). To understand the organization of cellular functions at different levels (gene, metabolite, or protein) and link them to a particular phenotype, an integrative approach is needed and is often referred to as 'systems biology' (Kitano, 2002, 2010). Biological systems are complex and cannot be understood by focusing on any one aspect of their highly interacting components. Because the functioning of a plant as a system concerns each of its molecular constituents (DNA, RNA, proteins, metabolites, ions, etc.), the expanding development of high-throughput data generation technologies made it possible to apply a systems biology paradigm in plant science. Large sets of comprehensive and quantitative data from plant samples grown under a wide variety of conditions have been produced. Massive databases from such high-throughput data have been created (Joyard and McCormick, 2010). The goal of systems biology is to understand how all these components function to bring about the observed phenotypes, and to elucidate the complete network of causes and effects from the molecule to the ecosystem. Identifying all the components of a single biological system is now within our means; however, assigning function to genes, proteins and metabolites is still a daunting task.

Major challenges include interpretation and integration of large datasets to understand the principles underlying the regulation of genes, metabolites and proteins, and how their combined interactions associate with variation in phenotype (Kim et al., 2010; Fukushima, 2009). Several attempts have been made to integrate multiple 'omics' data sets from different species. Even if we are still far from the initial objective of fully understanding how a given system works, is undeniable that the systematic analyses of the different 'omics' levels can facilitate the discovery of new candidate genes/QTLs and/or to assign functions to unknown proteins. Networks and pathways have been reconstructed using transcriptome, genome–wide transcription factor binding, proteome and metabolome data, and subsequently used to infer functional interactions among genes, proteins and metabolites (Moreno-Risueno et al., 2010). Moreover, systematic analyses of the transcriptome and metabolome and correlation of the expression pattern of genes with the accumulation pattern of metabolites have been successful ways to deduce the functions of genes.

The guilty-by-association principle states that a set of genes (or proteins and metabolites) involved in a certain biological process is generally co-regulated and thus co-expressed under the control of a shared regulatory system (Saito and Matsuda, 2010). Therefore, if an unknown gene is co-expressed with known genes of a particular biological process, researchers assume that this unknown gene may be involved in this process. This co-occurrence principle can be extended to metabolite co-accumulation relationships with the expression pattern of genes of the particular pathway in which the metabolite is involved.

Following the comprehension of the whole biological system under investigation, it is of fundamental importance to identify the hubs that regulates it, in order to focus on these key-elements that could be successfully transferred into new varieties by breeding schemes or genetic transformation. In this review, we focus on how different genome-wide datasets have been and can be used to reconstruct biological networks in tomato flesh fruit, and to dissect the QTLs that underlie their genetic control, reporting as example the approaches established on the combined use of different platforms available for tomato.

GENETIC AND GENOMIC TOMATO RESOURCES

Tomato has long served as a model system for plant genetics, development, physiology, pathology, and fleshy fruit ripening, resulting in the accumulation of substantial information regarding the biology of this economically important crop. Besides a large amount of already well-established genetic and genomic resources, today even more high-throughput datasets and different platforms have been generated. Among the tomato genetic resources, besides wild and related species publicly available at the Tomato Genetics Resource Center (TGRC) (http://tgrc.ucdavis.edu/index.cfm), different mutant collection (http://zamir.sgn.cornell.edu/mutants/ and http://tomatoma.nbrp.jp/) (Barone et al., 2009), and TILLING populations were developed in several countries (Minoia et al., 2010; Piron et al., 2010; Okabe et al., 2011). A powerful material to dissect genetic complex traits as quantitative trait loci (QTLs) is represented by the introgression line (IL) populations. These populations consist of a number of homozygous lines each containing marker defined segments from the wild genome in a uniform cultivated genetic background. They allow the same genetic stocks to be used worldwide in genetics and genomics applications for tomato breeding. Currently, different IL populations are available derived from wild tomato species such as *Solanum pennellii*, *Solanum habrochaites*, *Solanum pimpinellifolium*, *Solanum lycopersicoides*, *Solanum chmielewskii* and *Solanum sitiens* (Fernie et al., 2006). These lines have been widely used to localize QTLs on the molecular map, and to identify putative genes involved in their genetic control (Lippman et al., 2007). This has greatly helped the breeding work for these traits, which show a continuous variation and are strongly influenced by environmental conditions.

On the other hand, in addition to the recently tomato ge-

nome sequence (www.solgenomics.net, release SL2.40 January 2011), a large amount of genomic resources are now available. High-density genetic and physical maps, derived from interspecific crosses between *S. lycopersicum* and *S. pennellii*, *S. habrochaites*, *S. pimpinellifollium*, and other wild relatives have been developed (Foolad, 2007). Moreover, many EST collections (more than 330,000 EST deposited in the Tomato Gene Index database) deriving from various tomato species and tissues and different developmental stages are also publicly available. Different microarray platforms (TOM1, TOM2, combimatrix, affimetrix, and agilent) have been used to study the transcriptomic change in different tissues and at different environmental conditions (Alba et al., 2004, 2005; Di Matteo et al., 2010; Balaji et al., 2008; Lemaire-Chamley et al., 2005). A SolCAP chip (http://solcap.msu.edu) containing more than 8000 single nucleotide polymorphism (SNPs) has been made available for the tomato scientific community (Sim et al., 2012a). These SNPs were mainly discovered based on NGS-derived transcriptomic sequences obtained from six tomato accessions (Hamilton et al., 2012). Finally, very recently, a diversity array technology (DArT) platform for tomato using the *S. pennellii* ILs population has been developed and validated (Van Schalkwyk et al., 2012). These recent genomic resources add to other high-throughput genotyping platforms that are being used to explore the level of polymorphism detectable within cultivated tomato by genome-wide analysis (Sim et al., 2009, 2012b; Robbins et al., 2010; Shirasawa et al., 2010a).

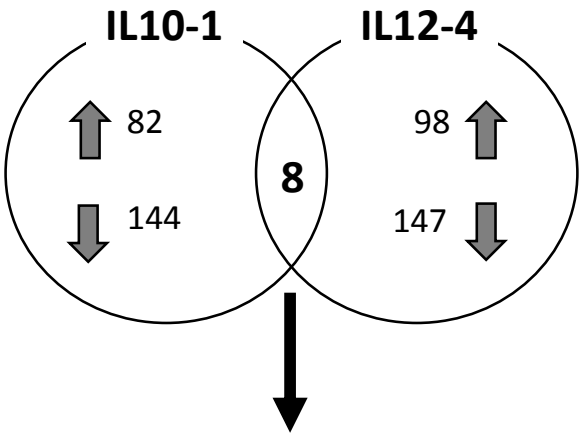
Alongside of genomic resources, there are an increasing number of powerful computational pipelines for sequence analysis and genome annotation. SGN (Solanaceae Genome Network, http://solgenomics.net/) is a website that provides a virtual workbench for researchers working on the *Solanaceae* family, which hosts various sources of data and analysis tools. The Metabolome Tomato Database (MoTo DB) is an open-access metabolome database for tomato fruit. The database was developed using fruits from 96 different tomato cultivars in different ripening stages ensuring a representative fruit sample. The Tomato Functional Genomics Database (TFGD, http://ted.bti.cornell.edu/) provides a comprehensive resource to store, query mine, analyse, visualize and integrate large-scale tomato functional genomics data sets (Fei et al., 2011). A web-based system (plant MetGenMAP) has also been developed, which can com-prehensively integrate and analyze large-scale gene expression and metabolite profile data sets along with diverse biological information (Joung et al., 2009). Other web resources that collect data generated from different tomato 'omics' approaches are publicly available and reviewed in Yano et al. (2007) and Barone et al. (2008).

A transcriptomic-based approach

Thanks to the tremendous technical advances of the post-genomics era, data generation is no longer the limiting factor in advancing biological research. In addition, data integration, analysis, and interpretation have become key bottlenecks and challenges that biologists conducting genomic research face daily. In the last few years, many reports in tomato research focused on the possibility of understanding the complex of fruit ripening (Alba et al., 2005; Kok et al., 2008; Palma et al., 2011) and nutritional quality composition (Shauer et al., 2006; Moco et al., 2006, 2008) by using different 'omics' approaches. In our lab, we undertook two different 'omics' approaches to in-depth understand molecular mechanisms underlying tomato quality traits with particular attention to the ascorbic acid (AA) synthesis and accumulation in the fruits. The first approach consists on the screening of a *S. pennellii* IL population (genomic level), for the ascorbic acid content (metabolic level) to detect QTLs controlling its synthesis and accumulation into the tomato fruits, and on comparing the microarray analyses (transcriptomic level) of the ILs carrying the specific QTL. We identified two genotypes, IL12-4 and IL10-1, carrying a QTL for higher and lower AA accumulation compared to the control M82, respectively. Surprisingly, the transcriptomic analyses of these ILs revealed that the genes directly involved in the main metabolic pathway of AA synthesis and recycling / catabolism showed not differentially expression with respect to the cultivated parent. Indeed, the higher AA content in the IL12-4 was supposed to be controlled through the up-regulation of genes driving pectin degradation, thus releasing intermediates for the L-galactonic acid pathway, which is an alternative biosynthetic pathway for AA synthesis in plant (Di Matteo et al., 2010) previously detected only in strawberry (Agius et al., 2003) and grapevine (Cruz-Rus et al., 2010).

In the IL10-1, the different expression of genes involved in carbohydrate catabolism, fatty acid biosynthesis, glyoxylate metabolism and antioxidant system were involved in a reduced AA level of tomato ripe fruit (Di Matteo et al., 2012). Therefore, in both cases, the combined use of the ILs with the microarray platform, allowed the identification of new genes candidate to the control of AA level in the tomato fruit. The identified differentially expressed transcripts were mapped onto the tomato genome available at the Solanaceae Genomics Network. The genetic positions were obtained by BLASTN (Altschul et al., 1990) searches against the entire Tomato WSG Chromosomes (SL2. 40) database (http://solgenomics.net/index.pl). Among the 20 genes of the IL12-4 model (Di Matteo et al., 2010), three mapped on the introgression 12-4, whereas in case of IL10-1, among the 17 genes which establish the model (Di Matteo et al., 2012), eight mapped to the introgression 10-1. Therefore, we can state that the transcriptomic analysis led to dissect the two QTLs for AA content that map to chromosomal regions 10-1 and 12-4 into candidate genes, whose specific function will be further investigated by other 'omics' approaches, such as the TILLING or VIGS platforms today avail-

a)



b)

TC	Chr	IL10-1	IL12-4	Annotation
TC189778	1	-	-	Unknown
TC170015	1	-	-	REF family protein
TC184006	2	+	+	Cystathionine gamma synthase
TC173750	4	+	+	NA
TC182944	10*	-	-	Serine/threonine protein phosphatase-related
TC182124	10*	-	+	Unknown
TC180762	10*	+	-	Translation elongation factor
TC180189	11	-	-	Unknown

Figure 1. a) Venn diagram showing the shared differentially expressed genes between the *Solanum pennellii* introgression lines (ILs) 12-4 and 10-1; b) list of the eight shared genes between the ILs 12-4 and 10-1.

available for tomato (Minoia et al., 2010; Orzaez et al., 2009).

In addition, it is interesting to note that candidate genes for AA control in tomato fruits of ILs 10-1 and 12-4 have also been found outside of the introgression regions, giving us a more complete picture of the genes and molecular mechanisms controlling metabolic pathways and their interactions. For example, out of these genes, 10 mapped to chromosome 1, five per each IL; for some of these genes, it is possible to hypothesize an interaction of 'key-elements' mapping on chromosome 1 with genes mapping on the regions 10-1 and 12-4, in controlling AA synthesis and accumulation in tomato fruit. As a whole, comparing the differentially expressed transcripts obtained from the two different microarray experiments (Figure 1), we found that a set of 8 transcripts were shared between the IL12-4 and the IL10-1, and among them two (TC182124 and TC180762) mapping on the introgressed region 10-1 also exhibit a contrasting expression pattern. One is annotated as a translation elongation factor p whereas the other is still not annotated. For the latter, a possible role as regulator of genes controlling AA level in tomato fruit could be hypothesized and further investigated in the future. These results highlight the powerful employment of the ILs in combination with the microarray approach.

Comprehensively, identified genes mapping within or outside the introgressed regions may represent key-control points in the mechanisms regulating the AA content in tomato fruit and so very useful in breeding program aimed to increasing nutritional quality in tomato fruits.

A genomic-based approach

The introgression lines as source of favorable alleles to transfer in the cultivated varieties have been so far used

Table 1. Characteristics of SNP assayed on the 96 tomato sample collection by the SolCAP genomic platform.

Missing value data point	Analyzed SNP (Number)	Segregating SNP (No.)		
		MAF = 0	MAF<10%	MAF>10%
MV = 0	6427	1410	3102	1915
MV<10%	1154	-	446	708
MV>10%	38	n.d.	n.d.	n.d.
Total	7619	1410	3548	2623

MV = Missing value. MAF=Minor frequency allele.

in tomato with the aim of identifying QTLs, both in conventional breeding (Lippman et al., 2007) and combined with 'omics' platforms (Schauer et al., 2006; Di Matteo et al., 2010, 2012). Despite these approaches allowed to identify many QTLs controlling very different traits, they allowed to explore reduced genetic backgrounds, generally limited to a few wild species (Fernie et al., 2006). By contrast, a wider source of genetic variation can be found among and within tomato breeding lines or cultivated varieties and ecotypes collected from different geographical regions. This is particularly true for traits important for adaptation to different environments. Most of this variation is of a quantitative nature and therefore requires specific genetic strategies for detecting QTLs, such as association mapping. In this case, large populations have to be phenotyped for the trait under study and genotyped through molecular markers uniformly distributed all over the genome, to perform a wide-genome approach of association mapping aimed at identifying new genes controlling the trait.

As an alternative, a candidate-gene approach could also be used, where variation at genomic level is specifically investigated for a number of genes already known to be involved in determining the phenotypic trait under study. In both cases, a high number of markers and/or genes already mapped on chromosomes are required. For tomato, a high-density map including different markers and genes is already available (Foolad, 2007; Shirasawa et al., 2010a, 2010b). Moreover, recently a genomic platform for SNPs detection has been built in the framework of the Solanaceae Coordinated Agricultural Project (SolCAP) from NIFA/USDA, based on the ILLUMINA Infinium Technology. The SolCAP tomato panel initially included around 8000 SNPs. These consist of Sanger-based eSNPs from genome sequences of two processing tomato lines (TA496 and Heinz 1706), besides those identified from ILLUMINA transcriptomic sequencing of three fresh-market lines, one processing line, one cherry tomato and one accession of the wild species *S. pimpinellifolium*. Therefore, depending on the germplasm assayed by the SolCAP genomic platform, various levels of polymorphism could be detected. A germplasm panel that consists of 489 accessions has been so far (September 27th, 2011) genotyped in the framework of the SolCAP activities (Sim et al., 2012b). This panel includes 141 accessions for processing, 122 accessions for fresh-market, 88 vintage tomatoes, 103 accessions belonging to various wild species and 35 accessions of miscellaneous materials (hybrids, F1 etc).

Data from this experiment are available for the scientific community (http://solcap.msu.edu) in order to compare the level of polymorphism detected in this population representative of genetic variability available among tomatoes with that highlighted in other specific tomato collections belonging to specific institutions, as already reported by Hamilton et al. (2012). In our laboratory, an association mapping approach by candidate gene has been undertaken with the aim of identifying among 96 different genotypes new alleles in genes that could increase the level of antioxidants in the fresh and processed fruit. In this context, a collection of *S. lycopersicum* accessions is being investigated for fruit quality traits in a 2-years trial in order to measure physiological and metabolic characters correlated with antioxidant synthesis and storage. In the meanwhile, the collection has been genotyped using the SolCAP platform for high-throughput genomic analysis. The collection under study mainly consists of Italian ecotypes (39 accessions), Latin American cultivars (29 accessions), some vintage cultivars and modern varieties coming from different geographical regions (that is, from Spain, China, Africa, USA). The variability exhibited by this collection, as evidenced by SNP analysis on the SolCAP genomic platform, resem-bles in the percentage that highlighted by the SolCAP experiment on the panel of 489 genotypes. Indeed, most of SNPs analyzed (98.2%, Table 1) had a missing value <10%, that means they lacked in less than 86 genotypes out of 96, and this value is similar to the 97.6% observed in the larger tomato population of 489 genotypes. The 38 SNPs that showed a missing value (MV, Table 1) higher that 10% were excluded by subsequent analyses.

Among others, a threshold value of minor frequency allele (MAF) of 10% was established. Overall, 1410 SNPs out of 7619 (18.5%) did not segregate among the 96 genotypes, since they only exhibited a single allele in all genotypes and no minor alleles were detected (MAF = 0). The distribution of all 7619 SNPs on the 12 tomato chromosomes was obtained by physically mapping them on the macromolecules obtained from the complete sequenced tomato genome (version 2.40 available at the

Table 2. Distribution over the 12 tomato chromosomes of SNP (with MV = 0 and MAF>10%) suitable for association mapping studies.

Allelic frequency	chr1	chr2	chr3	chr4	chr5	chr6	chr7	chr8	chr9	chr10	chr11	chr12	Total
10%<AF<15%	28	12	43	30	179	13	11	15	9	3	66	7	416
15%<AF<20%	10	28	39	179	207	27	12	14	8	3	111	19	657
20%<AF<25%	16	18	20	31	4	3	8	12	36	3	26	2	179
25%<AF<30%	22	25	33	19	5	9	5	9	20	9	65	8	229
30%<AF<35%	6	15	17	9	7	10	4	8	13	14	3	5	111
35%<AF<40%	7	7	6	20	6	2	10	15	10	7	5	8	103
40%<AF<45%	11	6	28	5	8	2	3	7	12	3	6	11	102
45%<AF<50%	30	4	9	8	5	2	9	10	9	1	4	14	105
Total	130	115	195	301	421	68	62	90	117	43	286	74	1902

AF = Allelic frequency; AF = 10% includes segregation ratios = 86:10; AF = 50% includes segregation ratios = 48:48; MV = missing value; MAF = minor frequence allele.

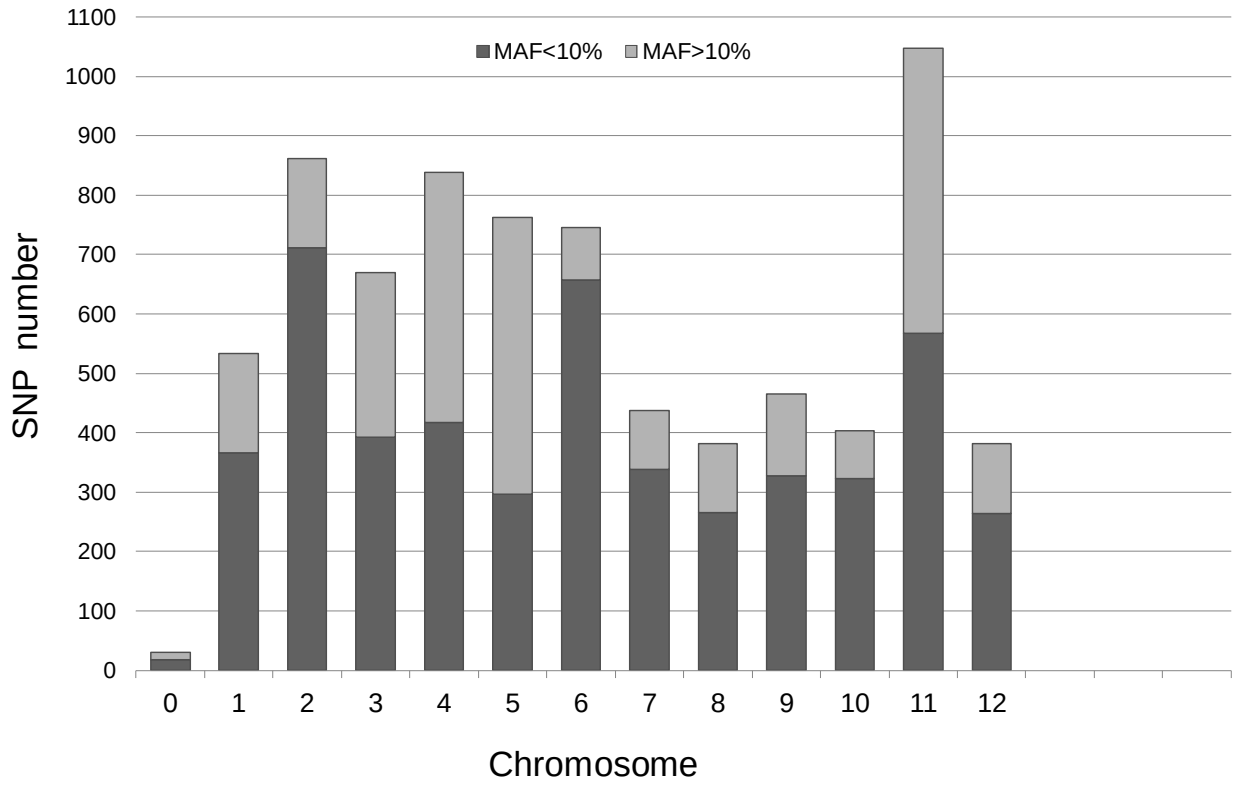


Figure 2. Distribution of 7619 SNPs on the 12 tomato chromosomes. MAF = Minor frequency allele.

http://solgenomics.net). The highest number of SNPs map on chromosome 11 (Figure 2), with an equal distribution between SNPs with MAF>10% and < 10%. A lower number of SNPs map on chromosomes from 7 to 12, whereas this number is higher for chromosomes from 1 to 6. In most cases, except than chromosomes 5 and 11, on each chromosome SNPs with MAF<10% are prevalent. Since many SNPs (3548 corresponding to 46.6%) fall within the group with MAF<10%, they should be mostly considered rare alleles (present only in one or two genotypes), and not useful for association mapping analysis. This high frequency of rare alleles, estimated considering the threshold value of 10%, confirms the one evidenced by Labate et al. (2009) who analyzed 30 tomato landraces by different types of markers. In our collection, the 2623 SNPs showing MV<10% and MAF>10% were the only suitable for the linkage disequilibrium analysis required to associate gene polymorphism with phenotype variation. They were located on the tomato molecular map (Figure 3) and it is evident that some chromosomes are better covered and that markers mainly cluster in specific areas of each chromosome, generally in distal ones. This would suggest extending our analysis to a greater number of genotypes and of markers, even though

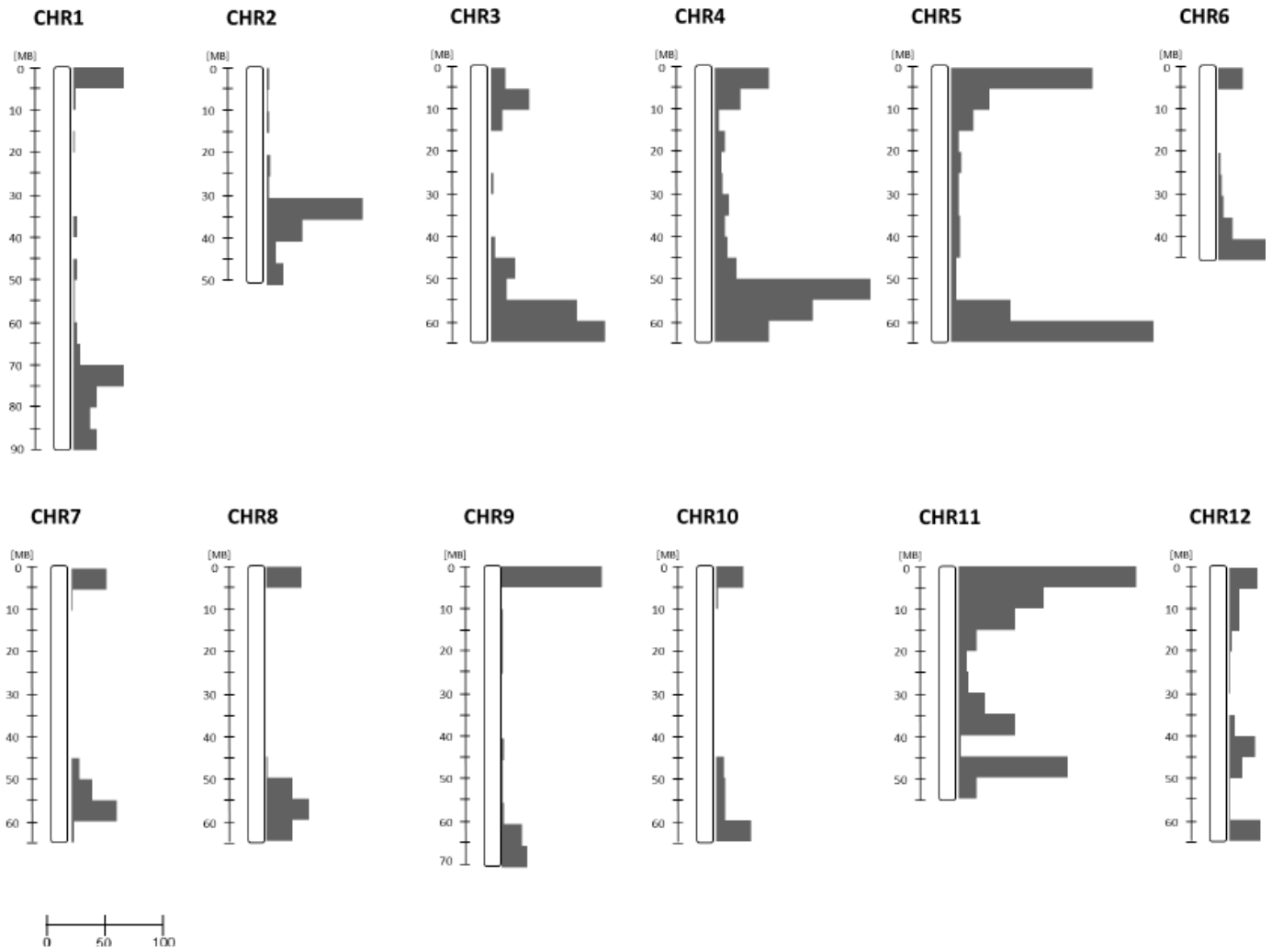


Figure 3. Mapping on tomato chromosomes of 2623 SNPs showing MV ≤ 10% and MAF > 10%. MV = missing value; MAF = minor allele frequency.

preliminary information could be derived for realizing a genome-wide association mapping approach.

Indeed, in order to explore the level of variability available in our collection, the segregation ratios observed for 1902 markers with MV = 0 and MAF>10%, distributed for each chromosome, are reported in Table 2, excluding those that still map on chromosome 0 or are not assigned to any chromosome (http://Solgenomics.net). Segregation ratios were grouped in eight different intervals, ranging from 10% (86:10) to 50% (48:48) of the allelic frequencies. Each interval includes five segregation ratios corresponding approximately to 5% of the allelic frequencies. Two intervals (10 to 15% and 15 to 20%) include about 50% of the markers (1073 SNPs of 1902) while the other ones include about 100 SNPs, except than the interval 25 to 30% (229 SNPs). These segregation ratios suggest that a higher variability is evident in our tomato collection compared to that exhibited by the collection of 30 accessions analyzed by Labate et al. (2009). Moreover, distribution of the 1902 SNP markers with MAF >10% on chromosomes shows that most of all map on chromosome 5 (421 SNPs) but also on chromosomes 4 (301 SNPs) and 11 (286 SNPs) while only 43 SNPs map on chromosome 10.

Consequently, in order to find new genes associated to AA variation by a genome-wide AM approach, genotypes should be added to our collection with the aim of further increasing the genetic variability to be explored. Finally, as for the candidate gene AM approach, based on the gene annotation actually available for the sequenced tomato genome, among the 7720 SNPs represented on the SolCAP platform, 28 genes belong to the metabolic pathways that lead to AA biosynthesis and accumulation (Ioannidi et al., 2009; Zou et al., 2006).

Among these, 25 segregate in our population of 96 genotypes, 17 as minor alleles (MAF<10%) whereas the other eight show a high level of variability and map on chromosomes 3, 4, 5 and 10. These polymorphisms will

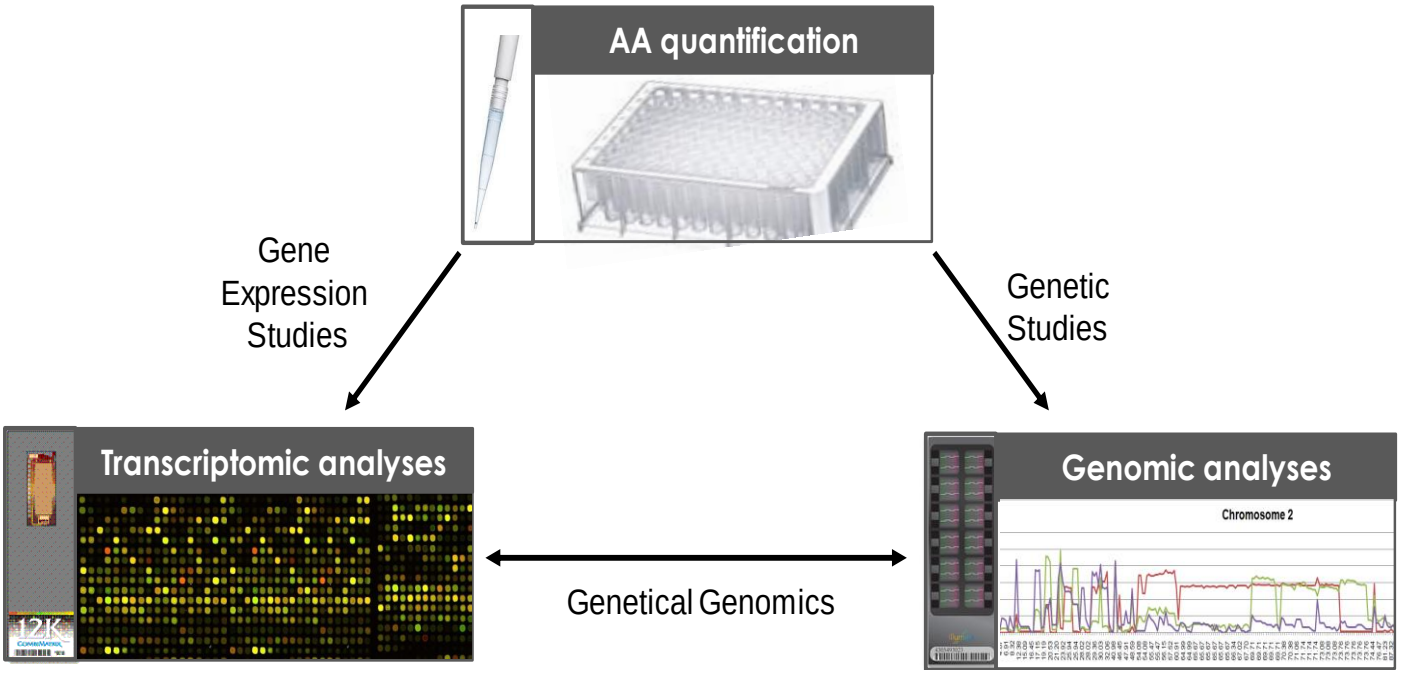


Figure 4. Integration of 'omics' data to improve and accelerate the production of new elite lines: combined transcriptomic and genomic analyses of different tomato lines will facilitate the identification of candidate genes for the trait of interest and will enhanced tomato breeding efficiency.

be further investigated referring to their potential association with different levels of AA content in tomato fruit. In the future, new alleles will also be searched for those candidate genes involved in AA level regulation that were identified by the transcriptomic approach here described. This high integration among 'omics' platforms will be extremely powerful in detecting genes or alleles up until now unexplored for the improvement of the nutritional quality of tomato fruit. It could be also successfully applied to other traits under the complex control of many genes/QTLs, thus allowing to identify genes/hubs and to realize breeding by precision approach in tomato (Collard and Mackill, 2008).

CONCLUSION

Our comprehension of complex molecular networks that underlie biological processes has grown dramatically in the last few years. In this review, we have highlighted the use of some 'omics' platforms and tools today available for tomato researches to better understand molecular mechanisms controlling fruit AA synthesis and storage. Our goal was to unveil new genes and their relations (transcriptomic-based approach) or new alleles (genomic-based approach) involved in modulating AA accumulation in tomato fruit in order to manipulate them for enhancing fruit antioxidant content. Comprehensively, this would lead to an approach of genetical genomics, as proposed by Kirst and You (2007), which incorporates the transcription level information at integration of those from genotyping and phenotyping to identify candidate genes for complex traits (Figure 4). The application of post-genomics tools should accelerate the selection process and the combined use of different 'omics' strategies and will considerably shorten the time required for the production of elite lines. Indeed, as genome sequencing becomes less costly and the development of the most recently technique, such as RNAseq (Wang et al., 2009), protein–DNA binding microarrays (Badis et al., 2009) and genome-wide profiling of histone modifications and DNA methylation (Lister et al., 2008; Zhang et al., 2009) is increasing, the comprehension of complex biological phenomena will certainly improve.

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Full Length Research Paper

Short hairpin RNA expression for enhancing the resistance of *Bombyx mori* (*Bm*) to nucleopolyhedrovirus *in vitro* and *in vivo*

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A new paradigm of RNAi technology has been studied for enhancing the resistance to virus in plants and animals. Previous studies have shown that the *Bombyx mori* (*Bm*) U6 promoter based shRNA is an effective tool for inducing RNAi in *Bombyx mori* cell line. However, widespread knockdown and induction of phenotypes in *Bm* larvae have not been fully demonstrated. In this study, we examined *Bm* U6 promoter based shRNA expression for suppressing *Bm* nucleopolyhedrovirus (NPV) in the *Bm* cell line and silkworm larvae. We measured the relative expression level of replication genes of *Bm*NPV in hemolymph of silkworm larvae and *Bm*N cells transfected with recombinant targeting shRNA by quantitative real time polymerase chain reaction (PCR). These results indicated that the recombinant shRNA expression system was a useful tool for resistance to *Bm*NPV *in vivo* and *in vitro*. The approach opens the door of RNAi technology as a wide range of strategies that offer a technically simpler, cheaper, and quicker gene-knockdown by recombinant shRNA for future genetics in silkworm *Bm* and other related species.

Key words: RNA interference (RNAi), Silkworm *Bombyx mori* (*Bm*) cell line, short hairpin RNA (shRNA), *Bm* nucleopolyhedrovirus (*Bm*NPV), quantitative real time polymerase chain reaction, *Bm* U6 promoter.

INTRODUCTION

RNA interference (RNAi) is the mechanism of introducing a small RNA into a cell to suppress the target gene's expression. RNAi technologies have been used as a highly useful genetic tool for therapeutic and specific knockdown of particular genes in mammals, invertebrates, and plants (Agrawal et al., 2003; Dawe, 2003; Fire et al., 1998). Characteristic feature of RNAi and antiviral role were first identified in plants. Successively, RNAi had been used as an antiviral curative in animal systems and a gene-therapeutic agent (Gitlin et al., 2002; Li et al., 2002; Sato et al., 2002). RNAi was induced in mammalian cells by the transient transfection of short dsRNA oligonucleotides (21- to 23- bp siRNA). Since the use of artificial siRNA does not lead to a long term effect, researchers developed plasmids encoding short hairpin RNA (shRNA), which are processed in the cell to generate siRNA (Arendt et al., 2003; Peng et al., 2007).

The *Bombyx mori* nucleopolyhedrovirus (*Bm*NPV) is one among the most destructive diseases in silkworm. Although scientists have developed resistance of *Bm* to *Bm*NPV through breeding, it is not completely effective. Control of *Bm*NPV in silkworm is a major factor of silk industry. *BmNPV* is an insect virus which is in baculoviridae family. It has a circular double stranded DNA of 130 kbp. Studies have shown that specific viral genes such as *P*143 and *P*35 are required for multipli-cation of viruses. These two genes were first identified in *Autographa californica* multiple nucleopolyhedrovirus

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(AcMNPV) (Marcel et al., 1994). *P*143 gene is encoded 143 kDa polypeptide with motifs conserved among DNA helicases. The presence of this gene is necessary for *Bm*NPV DNA replication. *P*35 gene is stimulated by DNA replication in AcMNPV. Lack of *P*143 and *P*35 genes in cells infected with NPV could be effective in control *Bm*NPV infections in silkworms.

Isobe et al. (2004) successfully suppressed the multiplication of *Bm*NPV *in vivo* when the *lef1* dsRNA was infected in *Bm*N cells. Using transgenic silkworms to transcribe ie1 dsRNA of *Bm*NPV by ie1 promoter decreased the lethality of *Bm*NPV in the silkworm larvae.

In this paper, we generated the targeting of shRNAs against *Bm*NPV and analyzed the efficiency of *Bm*NPV knock-down by shRNAi plasmid, which is regulated by a *Bm* U6 promoter with a marker gene of green fluorescence protein (GFP), in *Bm* cells and silkworm larvae. We selected the most efficient shRNAs for resistance to *Bm*NPV. The results suggested that this technique is useful for suppressing the *Bm*NPV both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Cell lines

The *Bm* ovary derived cell line (*Bm*N cells) was conserved in our lab and cultured on TC-100 medium (GIBCO, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum followed by incubation at 27°C.

Virus

*Bm*NPV was purified from strain T3 and propagated in the *Bm*N cell lines at 27°C.

Experimental animals

A hybrid strain of silkworm (Commercial name: Baiyu x Qiufeng) was used in this experiment. The larvae were reared with fresh mulberry leaves at 25 to 27°C.

Chemistry reagents

Lipofectamine-2000 was purchased from Invitrogen, Shanghai, China First-strand of cDNA synthesis ReverTra Ace® qPCR RT Kit was purchased from TOYOBO, Japan MMLV first-strand cDNA synthesis kit was purchased from Sangon Company, Shanghai, China, and genomic DNA extraction kit was purchased from Sangong, China.

Construction of shRNA-expression plasmid

The shRNA were constructed according to knockout RNAi systems user manual. We constructed shRNA plasmids in PXL-BACII vector (*Piggy*Bac transposition vector). Briefly, recombinant plasmid were transformed in *Escherichia coli* strain using ampicillin containing LB plate for growing colonies at 37°C overnight. After growing colonies, we picked a colony and place them again on the fresh 0.5 µg/ml ampicillin LB agar for overnight shaking. We confirmed by sequencing and digested by *Bam*H1 and *Eco*RI endonuclease enzymes.

Already, the PXL-BACII-EGFP-BmU6-shRNAs were constructed in our lab. We constructed nine types of shRNA of *P*143 and *P*35 genes such as *P*143A, *P*143B, *P*143C, *P*143D, *P*143E, *P*35A, *P*35B, *P*35C, and *P*35D. Enhanced green fluorescence protein (EGFP) was used as reporter plasmid.

Transfection of *Bm*N cells line

*Bm*N cells (1×10^6/well) were cultured on a 6-well plate. Each well was transfected with 5 µg of PXL-BACII-EGFP-*Bm*U6-shRNA. plasmid DNA using by Lipofectamine-2000 according to the manufacturer's instructions.

After 6 h incubation, the TC-100 medium with 10% fetal bovine serum (FBS) was replaced. After 24 h, each well was inoculated with 15 µl of *Bm*NPV (at a MOI of 3) and incubated at 26°C. From 24 to 72 h post transfection of plasmid, the GFP was observed under a fluorescent microscope (Nikon ECLIPSE Ti). The genomic DNA was isolated by TIANamp genomic DNA Kit (Tiangen Biotech, Beijing co., LTD.) 72 h post transfection of plasmid, and genes were confirmed by PCR using primers *Bm*ie1-f (5'-tcgacaacggctattcagag-3') and *Bm*ie1-r(5'-ctgcagtctcgctgtcagat-3').

Recombinant shRNA injected in silkworm larvae

The 5th instar first day of silkworm larvae were reared in six different groups. Each group's larvae were 50 pieces. First groups were normal as a negative control. Second groups were microinjected with 15 µl *Bm*NPV polyhedra (1×10^6/each larva) as a positive control. Other four groups were microinjected with same concentration and quantity of recombinant shRNA plasmid (5 µg) with lipofectamine-2000. After 24 h post infection, the larvae were challenged with *Bm*NPV (1×10^6/each larva). Observations were conducted day by day. After 96 h post infection, the hemolymph was collected in an eppendorf tube, and then centrifuged at 1000 rpm for 10 min.

RNA extraction and cDNA synthesis from *Bm*N cell and hemolymph

The total RNA was extracted from 96 h post infected cell samples using RNAiso Plus (TaKaRa Biotechnology Co., Ltd. China). We followed the same methodology for extraction of RNA from 96 h post infection of silkworm samples. The RNA was used as a template for first-strand of cDNA synthesis using ReverTra Ace® qPCR RT Kit (TOYOBO, Japan) under reaction conditions of 5 min at 65°C, 15 min at 37°C, 10 min at 98°C. For gene fragment amplification using actin A3 forward primer 5'-GCGCGGCTACTCGTTCACTACC-3' and reverse primer 5'-GGATGTCCACGTCGCACTTCA-3'. The PCR was conducted in a volume of 20 µl containing 7 µl double distilled water, 10 µl premix Ex Taq and 1 µl 10 mmol/1 primer F, 1 µl 10 mmol/1 primer R and 1 µl cDNA. The conditions of reaction were set as 30 s at 94°C, 30 s at 60°C, 1 min at 72°C and final extension for 10 min at 72°C.

Quantification of polyhedra of silkworm larvae

Hemolymph were harvested from single silkworm larva injected with *Bm*NPV as a positive control and simultaneously, hemolymph were harvested from larva injected with recombinant shRNA-plasmid at 48 and 96 h post infection, and centrifuged at 12000 rpm for 5 min. After which piled was collected and solubilized in 0.1% SDS in PBS buffer (pH 7.0) for 30 min at room temperature. The solubilized polyhedra were diluted 10 times with purified water. The larval hemolymph was checked for the presence of polyhedra under a hemocytometer at 48 and 96 h post infection. Each experiment was done in three replications.

Table 1. Synthesized shRNA sequences targeting the regions of the *p*143 and *p*35 of *Bm*NPV.

Construct	Position	Sequence
*p*143A	329-347	GGACTATTGTTGGTGCTCA
*p*143B	538-556	GGCAAACTTAACGCTGTCT
*p*143C	606-624	GTCATAATCGTCCACGTAC
*p*143D	1100-1118	TGCGCATGTAGAATCGAGT
*p*143E	1731-1749	GTTTAACGCGACTCGCATA
*p*35A	64-82	GACGAACAAACCAGAGAGT
*p*35B	117-135	GACAAAACCCGTTCTCATG
*p*35C	585-603	GTCTTAGCTTACGTGGACA
*p*35D	225-243	GATCAACTAGAACGCGAAT

Scanning electron microscopy (SEM)

The polyhedra were allowed to settle on the surface on to carbon-coated grids for 2 min. The grids were washed three times with distill water and stained with 2% uranyl acetate. The grids were examined using a Nikon Eclipse ME600 electron microscope (Nikon, Japan).

SYBR real-time quantitative PCR

PCR amplifications were performed using a 7500 fast real-time PCR System. The PCR was conducted in 25 µl system containing 6.8 µl double distilled water, SYBR premix Ex Taq 10 µl, 1 µl 10 mmol/1 primer F, 1 µl 10 mmol/1 primer R, 1 µl cDNA and ROX Reference Dye 0.4 µl (ROX) qPCR was completed according to protocol. Relative values of the genes expression data were calculated by Microsoft Excel.

RESULTS

Selection of shRNA and construction of recombinant plasmids

A total of 9 targeted sequences in the coding regions of *p*143 and *p*35 of *Bm*NPV were selected and served as a basis for the design of the complementary shRNA template oligonucleotides (Table 1).

The shRNAs were synthesized, annealed and inserted into the *Bam*HI and *Eco*RI sites of the siRNA expression vector pRNAi-Ready pSIREN-RetroQ-ZsGreen (Clontech). The ZsGreen and Neo resistant genes were amplified and sub-cloned into the vector PXL-BACII (*Piggy*Bac transposition vector. The *Bm*NPV targeting shRNAs were cut out from the above recombinant pSIREN with restriction sites *Bgl* II and *Eco*R I and the above constructed sub-cloned *piggy*Bac vector was designated as PXL-BACII-EGFP- *Bm*U6-shRNA.

Purification of polyhedra of silkworm

The single silkworm larva was injected with *Bm*NPV with 15 µl of *Bm*NPV (at a MOI of 3). After 96 h post infection, the hemolymph was harvested from larva and solubilized in 0.1% SDS in PBS buffer (pH 7.0). The polyhedra were identified under electronic microscope (Figure 1).

Viral challenge and investigation of interference against BmNPV *in vitro*

We investigated whether shRNA expression plasmid constructed by our method can induce gene expression in *Bm* cell line and larvae. The recombinant shRNA expression plasmid together with lipofectamine-2000 was co-transfected into the *Bm*N Cell and silkworm larvae.

After transfection of shRNA expression plasmid DNA (5 µg) containing lipofectamine-2000 in *Bm*N cell, all cells were clearly visualized for EGFP after 24 h when the cells were transfected with recombinant shRNA plasmid, and challenged with *Bm*NPV polyhedra (1×10^6). Observation suggested that almost 90% cells were transfected and EGFP was visualized. The *Bm*NPV challenge experiment showed that different shRNA has different suppression effect against *Bm*NPV in cells. Among them shRNA targeting *p*143E was most effective (Figure 2).

After challenge with *Bm*NPV, transfected shRNA with lipofectamine-2000 in cell was observed. The different suppression rates depend on different shRNA sequences.

Silkworm larvae challenged by *Bm*NPV

We chose best sequences for further experiments in the silkworm larvae. We selected four shRNAs (*p*143E, *p*35A, *p*35B, *p*35C) which were more suppressed from Figure 2. Four days after *Bm*NPV was infected; silkworm were observed to die (Figure 3B).

However, *P*143E shRNA infected silkworm was able to survive up to 6 days in comparison to infected positive control (Figure 3F). It was interesting to find that ShRNA infected silkworms were more active than *Bm*NPV infected silkworm and could be easily distinguished with

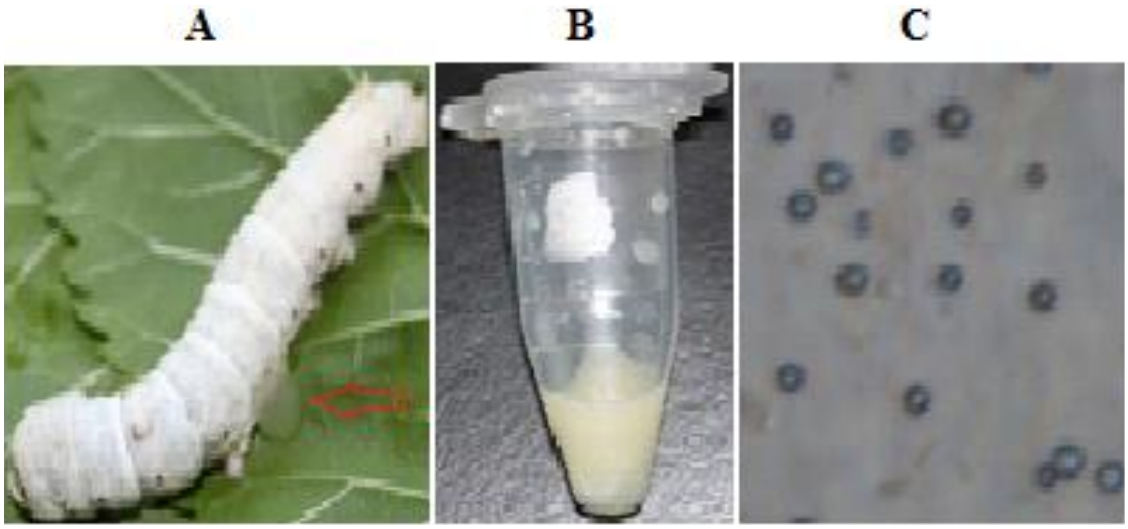

Figure 1. Purification of polyhedra of silkworm. A) Collect hemolymph from the silkworm. B) Purification of the hemolymph with 0.1% SDS in PBS (pH 7.0). C) Polyhedra of the *Bm*NPV under electronic microscope.

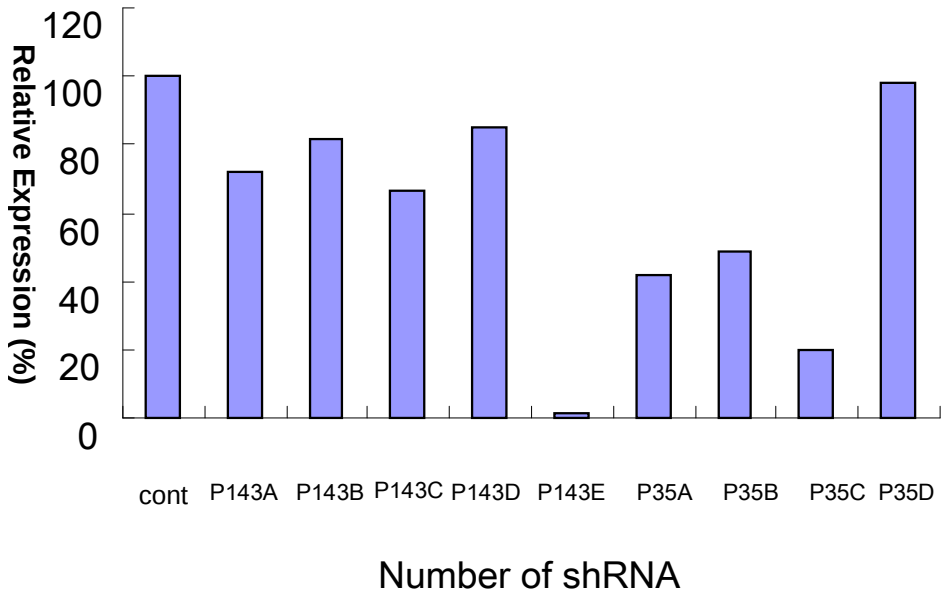

Figure 2. The target sequence-dependent suppression by shRNAs with U6 promoter in *Bm*N cells analysis by quantitative real- time PCR.

phenotypic characters. Some of the phenotypic traits were blackening of the silkworm body, loss of appetite, growth retardation, early maturing, swelling of the body and hemolymph becoming whitish (Figure 3).

Further, we examined the density of *Bm*NPV polyhedra in the infected hemolymph in each larva and then compared by hemacytometer under a microscope (Figure 4). We observed that *P*35C shRNA has less polyhedra (than positive control *Bm*NPV infected silkworm. We observed two different times at 48 and 96 hpi, the results were not same and varied with time period. The results indicated that *P*35C is less polyhedral than other infection of shRNA and also showed that the polyhedra were less than *Bm*NPV infected silkworm (Table 2).

Gene expression analysis

Figure 5 indicates that *p*35C is the most effective suppression of all. Though *p*143E shows the least suppression rate here, their suppression rate shows better in *Bm*N cell lines. Our findings demonstrate that at 5 µg

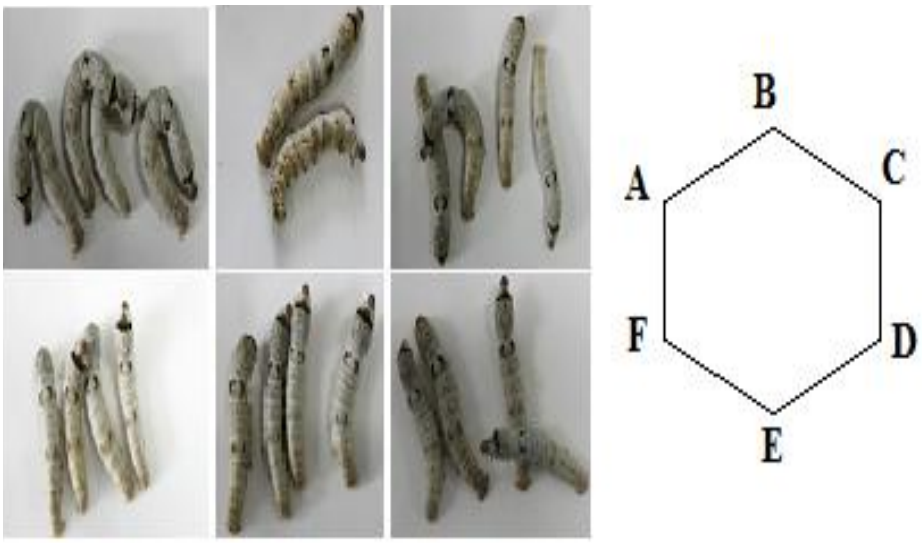

Figure 3. *Bm*NPV challenged with different recombinant shRNA *in vivo* after 96 hpi. A) Normal silkworm larvae (negative control), B) *Bm* larvae infected with *Bm*NPV, C) *Bm*NPV challenged with *P*35A shRNA, D) *Bm*NPV challenged with *P*35B shRNA, E) *Bm*NPV challenged with *P*35C shRNA, F) *Bm*NPV challenged with *P*143E shRNA. The silkworm was injected with *Bm*NPV polyhedra (1×10^5/each larva).

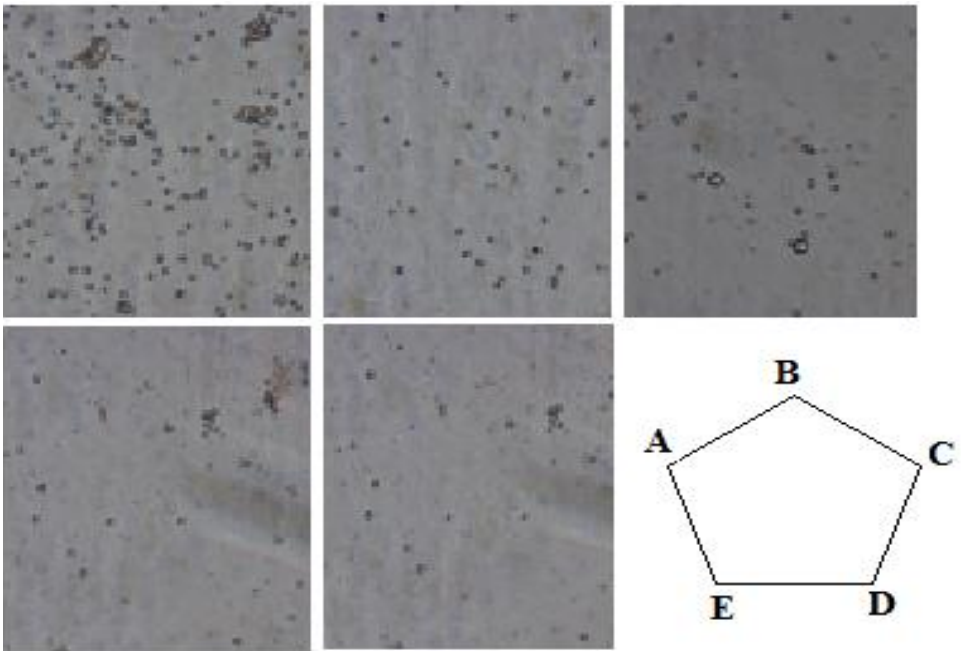

Figure 4. Density of polyhedra different shRNA treated silkworm hemolymph. A) *Bm*NPV infected silkworm hemolymph, B) *Bm* NPV challenge with *P*35A shRNA, C) *Bm* NPV challenge with *P*143B, D) *Bm* NPV challenge with *p*143E shRNA, E) *Bm* NPV challenge with *p*35C shRNA.

shRNA doses of injection to all silkworm larvae and *Bm*N cell lines, the level of expression varies, which indicates the variation mechanism involved *in vivo* and *in vitro* conditions.

*P*143 ShRNA is less suppressing than others. The result shows that in the *Bm* cell line *P*143E shRNA was

Table 2. Observation of *Bm*NPV polyhedron in hemolymph of silkworms at different times under hemocytometer microscope.

ShRNA name	Polyhedron observed (No.) (48 h post-injection)	Polyhedron observed (No.) (96 h post-injection)
Hemolymph of silkworm	0	0
NPV with silkworm	80	100
Bm NPV challenge with *P*35A	49	68
Bm NPV challenge with *P*35B	59	80
Bm NPV challenge with *P*143E	57	70
Bm NPV challenge with *P*35C	40	50

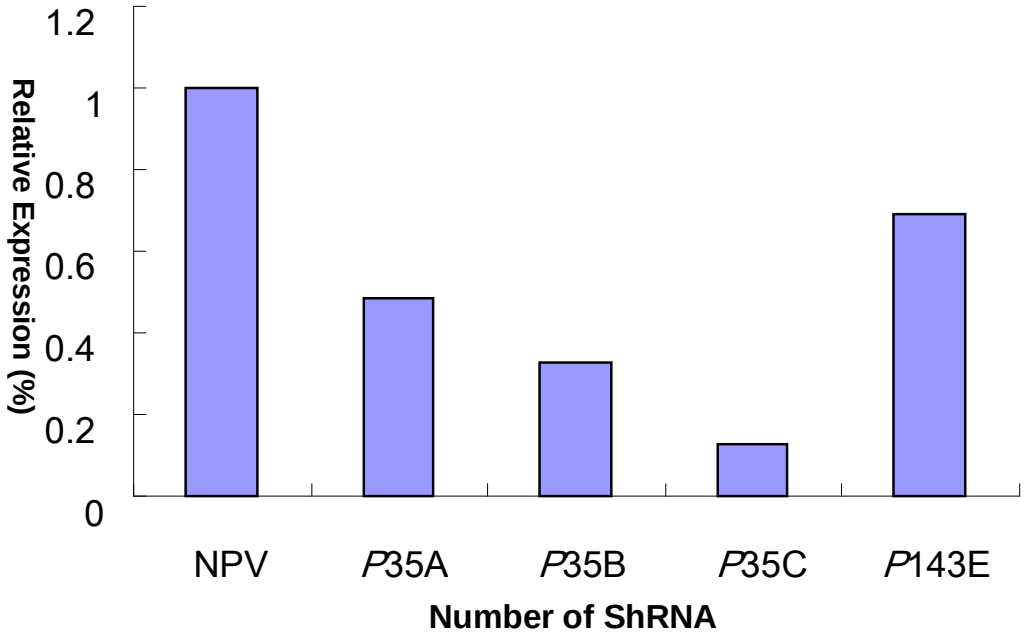

Figure 5. Relative expression analysis of shRNA in silkworm hemolymph post 96 h transfection. Indicated are: *Bm*NPV = Control larvae; *p*35A, *p*35B, *p*35C and *p*143E = Challenged *Bm*NPV.

more suppressive but in the organism, *P35C* is the most suppressive shRNA. Our findings demonstrate that with same amount of shRNA the suppression of the gene also differs in the organism. This behavior may be due to differences *in vivo* and *in vitro* mechanisms involved. The results showed almost similar suppression rate *in vivo* and *in vitro* conditions.

The *p*143 and *p*35 genes were identified by PCR. The samples were collected from infected larvae. The results showed that lane 2 was a negative control and does not have a band; lane 3 was a positive control and has a band; lanes 4 to 6, the *p*35A, *p*35B, *p*35C challenged with *Bm*NPV, also have a band of 430 bp; lane 7 was a positive control and has a band and lane 8 *p*143E challenged with *Bm*NPV and has a band of 275 bp. We confirmed from this picture that the *p*143 and *p*35 genes were presented in infected silkworm larvae (Figure 6).

DISCUSSION

RNAi is a promising tool for studying gene silencing in all eukaryotes. DsRNA duplex can suppress the expression of target gene through either mRNA degradation or blocking mRNA translation (Mcmamus and Sharp, 2002). shRNAs can be generated by an oligonucleotide DNA sequence. The shRNA constructs can trigger siRNA molecules to introduce the gene-specific silencing. Each shRNA vector system has the ability to silence specific gene. It has been demonstrated that *piggy*Bac2 vector can be successfully used for shRNA expression. This vector can also be linearized and as such; be ready for ligation and direct use in transient transfection experiments. Sequence encryption shRNA is a 19 to 21 bp of homology to the targeted gene and are synthesized as 60 to 75 bp double stranded DNA oligonucleotides

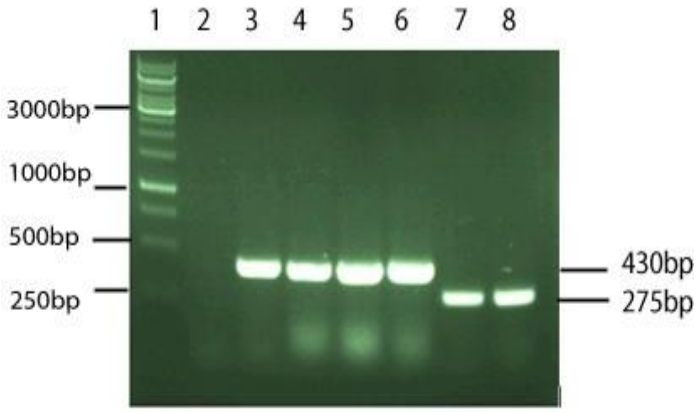

Figure 6. *P35* gene and *p*143 gene detection by PCR after injected recombinant plasmid challenged with *Bm*NPV in silkworm larvae. The genomic DNA as a template from hemolymph after 96 hr. infection. Lane1: DNA marker Lane 2: Control silkworm Lane 3: *Bm*NPV infected silkworm. Lane 4 to 6: p35 gene challenged *Bm*NPV. Lane7: *Bm*NPV infected silkworm Lane 8: *p*143 gene challenged with *Bm*NPV

(Patrick et al., 2002).

Recently, several works have been done on improving the pol lll expression system which successfully knock-down the gene expression (Isobe et al., 2004). However, there is need to improve this system for control of *Bm*NPV both *in vivo* and *in vitro* condition. Recently, dsRNA has been used for resistance *Bm*NPV in the silkworm by pol ll promotor (Ohtsuka et al., 2008). *Bm*NPV gene was silenced by small RNA using poll lll promoter; although, it is necessary to improve this technology for successful sequence-specific gene silencing in the *in vivo* and *in vitro* condition.

In this work, the selected *Bm*U6 promoter was used as a best tool for suppression of *Bm*NPV in the silkworm. We targeted five and four different positions of the genes *p*143 and *p*35, respectively (*p*143A, *p*143B, *p*143C, *p*143D, *p*143E and *p*35A, *p*35B, *p*35C, *p*35D) (Table.1). The shRNA presents a 7 to 9 nucleotide hairpin loop (5'-TTCAAGAGA-3') and the 19 base pair antisense sequence of the target site. The knock down efficiency of shRNA expression was different in *Bm* cell. The most effective suppression was observed in the plasmid targeting *p*143E gene (Figure 1, *p*143E) which suppressed the expression to about 90%, and *p*35C gene which suppressed the expression to about 70% (Figure 1, *p*35C). This result suggests that gene suppressing differs on the suitable sequence.

Further, we examined the density of *Bm*NPV polyhedra in the infected hemolymph in each larva transfected with both of *BM*NPV and shRNA plasmids. The results showed that *P*35C is less polyhedral than other infection of shRNA and also showed that the polyhedra were less than *Bm*NPV infected silkworm (Figure 4 and Table 2).

We examined relative expression level of the replicated *Bm*NPV genes in silkworm larvae, hemolymph and *Bm*N cells. Some of the shRNA worked successfully and showed less multiplication of *Bm*NPV. This RNAi system will be useful for suppression of *Bm*NPV in silkworm. It might eradicate *Bm*NPV in silkworm body in the future which will be valuable for silk industry.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Composition of raw cow milk and artisanal yoghurt collected in Maroua (Cameroon)

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The composition of milk is of most importance to the dairy industry and human health. This study was conducted to provide data on the composition of raw cow milk and artisanal yoghurt collected in Maroua (Cameroon). Milk and yoghurt samples were collected from 11 breeding sites and 12 producers in the city of Maroua, respectively. The following parameters were determined: pH, dry matter, ash, fat, lactose, total protein, non-casein nitrogen (NCN), non-protein nitrogen (NPN), true protein, whey protein, casein, amino acid composition, α-lactalbumin, β-lactoglobulin, αS1-casein, αS2-casein, β-casein, κ-casein and mineral composition. The results showed that, the composition of the milk and yoghurt varied from one sample to another. The chemical composition of some of the milk and yoghurt studied differed from the corresponding samples in previous studies. For example the mean pH of the raw cow milk (6.25) and artisanal yoghurt (3.84) were lower. The mean NPN levels of the cow milk and yoghurt (0.21g/100g) were higher. The mean fat content of milk (4.48 g/100 g) was higher. The protein fraction was lower in yoghurt while Fe, Cu and Mn levels were lower in both cow milk and yoghurt. The data reported in this paper would be helpful in dairy technology and public health.

Key words: Composition, cow milk, artisanal yoghurt, Maroua, Cameroon.

INTRODUCTION

Milk is a translucent white liquid produced by the mammary glands of mammals (William and Bowen, 2005). Milk and milk products are excellent high quality foods, providing nutrients (Aboul et al., 2005). They have an important place in the human diet. Milk has been described as being almost a complete food for man. It contains carbohydrates, protein, fats, vitamins, mineral elements and water. The quality and the composition of milk are very important to the dairy industry and human health because milk composition is related to milk process ability (Ozrenk and Selcuk Inci, 2008).

For centuries, milk production in Cameroon has been characterized by a traditional system using local zebu cows (Goudali, White Fulani and Red Fulani). The total milk production is estimated at 174000 tonnes/year (MINEPIA, 2009). It varies from 0.5 (in the dry season) to 3 L per day in the rainy season and per cow on a 180-day lactation period (MINEPIA, 2009). The total consumption

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Abbreviations: TN, Total nitrogen; **NCN,** non-casein nitrogen; **NPN,** non-protein nitrogen.

of milk and dairy products in Cameroon is estimated at 297,000 tonnes/year. Per capita annual consumption was 19.8 Kg in 2009. The production of cow milk in the Far North Region of Cameroon is 41 760 tonnes/year (ACDIC, 2006). Milk collection is difficult, due to the dispersion and remoteness of farms to the industrial processing units. Infrastructure for collection, storage and transport are almost inexistent. These infrastructural problems result to the deterioration of fresh milk before entering processing areas and even consumption, reducing revenues and profitability of the business.

Milk of lactating cows are often consumed in the country or incorporated in the preparation of maize, millet/sorghum and rice porridge. A large part of the milk is transformed into artisanal yoghurt by different Common Initiative Groups settled in the area. Several authors from various countries have studies on the composition of cow milk. The composition of cow milk varies considerably due to a number of factors: season, diet, age, stage of lactation, physiological status, genetic, environmental and region of production (Iggman et al., 2003; Farah et al., 2006; Slots et al., 2009; Mapekula et al., 2011; Frelich et al. 2012; Myburgh et al., 2012). The results obtained are specific to the country of study.

In Cameroon, several studies have been carried out on the composition of foods and dishes (Ponka et al., 2006; Fokou et al., 2009), but the composition of milk and dairy products has received little attention. Therefore, the aim of this study was to determine the composition of raw cow milk and artisanal yoghurt collected in Maroua, a town in the Far North Region of Cameroon.

MATERIALS AND METHODS

Collection area

The study area was Maroua, a town in the Far North region of Cameroon. This town is located between latitude 10 and 13° North and between longitude 13 and 16° East. Its population is estimated at about 200 000 inhabitants (RGPH, 2010). The climate is tropical Sudano-Sahelian type with two seasons: a long dry season of about eight months, from October to May and a short rainy season of four months, from June to September. Rainfall is low with an average precipitation between 900 and 1000 mm per year. Temperatures range from 17°C in November to 40°C in April. The vegetation is mainly thorny steppe and carpet grasses or ephemeral. The soils are clayey-sandy black or greyish (Donfack et al., 1996). The main activities are agriculture (with the cultivation of millet, corn, sorghum, cotton, vegetables among others), livestock (cattle, goats, swine, and poultry farming), crafts (jewellery, tannery) and trade.

Sampling

Surveys were conducted in Maroua city to identify cow' breeding sites and structures of artisanal yoghurt production. Fresh raw cow milk samples were therefore collected from 11 breeding sites: Hodango (M1), Medenguer (M2), Dabai (M3), Tondewo (M4), Mayelbehi (M5), Palama (M6), Doubazao (M7), Palaodi (M8), Lougadadou (M9). Hardé (M10), Doulgo (M11). Each of the 11 fresh milk samples was packaged in sterile bottles and transferred immediately to laboratory in the bucket containing ice because there was no cold chain for the storage of milk in the breeding sites. The 11 breeding sites had the same characteristics. In each site, there was a mixture of Goudali, White Fulani and Red Fulani cows. All the cows were thin. Milking was done by hand. Ages calves varied from 1 to 12 months. Lactating cows were fed essentially with millet pellicles, millet stalks, grass, salt and cotton crab. The only difference was observed with the amount of food served to cows per site.

Artisanal yoghurt samples were collected from 12 producers in the city of Maroua and labelled YA, YB, YC, YD, YE, YF, YG, YH, YI, YJ, YK and YL. For the manufacture of yoghurt, each producer used as ingredients a mixture of raw cow milk obtained from farmers of Maroua city, industrial yoghurt as a starter source and sugar in different proportions. For yoghurt preparation, whole cow milk was heated to boiling to destroy the pathogenic organisms at 80°C for 10 min. It was then transferred to a container and cooled to 40°C. Industrial Dolait yoghurt was added as a starter source (*Lactobacillus bulgaricus* and *Streptococcus thermophilis).* The proportion of sugar used is in general 1 kg for 10 l of milk. Once the starter, was completely mixed, it was then incubated at 41-43°C for 6-8 h to complete the preparation. Yoghurt is cooled and stored under normal refrigeration conditions. The milk collected in each site was a mixture of milk from the milking of several lactating cows. In the laboratory, each of the 11 milk samples and 12 artisanal yoghurt samples was packaged in sterile bottles and stored at -20°C, until analysis.

Chemical analysis

pH and proximate composition

pH was measured using an H1 9024Microcomputer pH meter (Hanna Instruments, Portugal). Dry matter was determined by drying 5 g of sample at 103°C for 7 h in a capsule containing sand according to IDF (1987). Fat was determined according to IDF (1997). Ash was determined after incineration of sample at 550°C for 7 h according to IDF (1964a), and total nitrogen (TN) content of sample, non-casein nitrogen (NCN) and non-protein nitrogen (NPN) fractions were prepared according to IDF (1964b). For NCN, the sample was acidified to pH 4.6 with a mixture of 10% (v/v) acetic acid and 1 M acetate buffer. For NPN, about 40 ml of 15% (v/v) trichloroacetic acid were added to 10 ml of milk. NCN and NPN samples were filtered through Whatman papers (Whatman Int. Ltd., Maidstone, UK) No. 42 and 40, respectively. TN, NCN and NPN were determined by the Kjeldhal method (IDF standard 20B 1993). Nitrogen content was converted into equivalent protein content using 6.38, 6.25 and 6.19 as conversion factors for TN, NCN and NPN contents, respectively (Karman and Van Boekel, 1986). Whey protein was calculated from the difference between NCN and NPN, casein N from TN and NCN, and true protein from TN and NPN, respectively.

Amino acid analysis

Total amino acid content was determined after hydrolysis of samples by 6 N hydrochloric acid at 110°C for 24 h in a vacuum sealed glass tubes according to Davies and Thomas (1973). The amino acid analysis of the hydrolysed samples was then carried out by cation exchange chromatography on a Biochrom 30 automatic amino acid analyzer (Biochrom Ltd, Cambridge, G.B.) according to Spackman et al. (1958) using lithium citrate buffers as eluants and ninhydrin post-column reaction system. Tryptophan was not determined.

Dairy protein quantification

α-Lactalbumin and β-Lactoglobulin contents were determined on

Table 1. pH and proximate composition of raw cow milk (g/100g) collected in Maroua (Cameroon).

Sample	pH	Dry matter	Ash	Fat	Lactose	Total protein	NPN	NCN	True protein	Whey protein	Casein
M1	6.12 ± .01^{g}	14.07±0.09^{d}	0.67±0.02^{abc}	5.60±0.10^{d}	6.19±0.07^{a}	3.33±0.06^{c}	0.16±0.00^{g}	0.69±0.00^{i}	3.17±0.06^{d}	0.53±0.00^{fg}	2.64±0.06^{c}
M2	6.41 ± 0.00^{d}	11.40±0.06^{h}	0.70±0.02^{abc}	3.20±0.10^{g}	3.96±0.06^{e}	2.91±0.01^{f}	0.19±0.00^{ef}	0.76±0.00^{f}	2.72±0.01^{g}	0.57±0.00^{e}	2.15±0.01^{f}
M3	6.58 ± 0.01^{b}	11.92±0.03^{g}	0.70±0.06^{abc}	3.05±0.05^{h}	4.02±0.02^{e}	4.26±0.02^{a}	0.23±0.00^{c}	1.07±0.02^{c}	4.03±0.02^{a}	0.84±0.00^{b}	3.19±0.00^{a}
M4	5.86±0.01^{i}	11.20±0.01^{i}	0.58±0.05^{def}	2.78±0.02^{i}	4.54±0.04^{c}	3.29±0.01^{c}	0.18±0.00^{f}	0.70±0.00^{i}	3.11±0.01^{de}	0.52±0.00^{g}	2.59±0.01^{c}
M5	6.03±0.01^{h}	12.49±0.06^{e}	0.62±0.01^{cd}	3.83±0.03^{f}	4.53±0.02^{c}	3.17±0.02^{d}	0.15±0.00^{g}	0.70±0.00^{i}	3.02±0.02^{e}	0.55±0.00^{ef}	2.47±0.02^{d}
M6	6.32±0.00^{e}	12.20±0.01^{f}	0.71±0.02^{a}	3.00±0.00^{h}	4.70±0.05^{c}	3.66±0.01^{b}	0.32±0.00^{a}	1.20±0.01^{a}	3.34±0.01^{c}	0.88±0.00^{a}	2.46±0.02^{d}
M7	6.26±0.01^{f}	11.29±0.07^{hi}	0.50±0.00^{f}	4.08±0.02^{e}	2.93±0.11^{g}	2.64±0.03^{g}	0.21±0.00^{d}	1.00±0.00^{d}	2.43±0.03^{h}	0.79±0.00^{c}	1.64±0.03^{h}
M8	6.72±0.01^{a}	11.84±0.09^{g}	0.68±0.03^{abc}	3.78±0.02^{f}	5.14±0.13^{b}	3.05±0.04^{e}	0.28±0.00^{b}	1.14±0.00^{b}	2.77±0.04^{g}	0.86±0.00^{ab}	1.91±0.04^{g}
M9	6.11±0.01^{g}	16.12±0.03^{b}	0.63±0.02^{bcd}	6.60±0.00^{b}	4.31±0.00^{d}	3.64±0.03^{b}	0.18±0.00^{f}	0.73±0.00^{h}	3.46±0.03^{b}	0.55±0.00^{ef}	2.91±0.03^{b}
M10	6.48±0.01^{c}	14.78±0.00^{c}	0.58±0.02^{def}	5.80±0.00^{c}	3.91±0.03^{e}	2.91±0.01^{f}	0.23±0.00^{c}	0.74±0.00^{gh}	2.68±0.01^{g}	0.51±0.00^{g}	2.17±0.00^{ef}
M11	5.85±0.01^{i}	16.37±0.02^{a}	0.54±0.00^{ef}	7.60±0.00^{a}	3.22±0.06^{f}	3.07±0.07^{e}	0.20±0.00^{d}	0.83±0.01^{e}	2.87±0.07^{f}	0.63±0.00^{d}	2.25±0.06^{e}
Mean	6.25±0.27	13.06±1.87	0.63±0.07	4.48±1.57	4.31±0.86	3.26±0.43	0.21±0.05	0.87±0.19	3.05±0.43	0.66±0.15	2.40±0.42

NCN, Non-casein nitrogen ; NPN, non-protein nitrogen. Mean values in the same column with different superscript letters are significantly different (P< 0.05).

the filtrates obtained after acid precipitation of caseins at pH 4.6 (NCN filtrates). These extracts were further acidified to pH 2 by dilution in a trifluoroacetic acid solution (final concentration: 0.1 %). The analysis was then carried out using a Dionex ICS 3000 HPLC system (Thermo Fisher S.A., Voisins le Bretonneux, France) by reverse phase separation on a C4 Vydac (214TP5415) type column. The acetonitrile gradient for elution was 37 to 50 % for 15 min at 40°C and the absorbance was measured at 214 nm in a Dionex Ultimate 3400 RS Variable Wavelength Detector. The caseins: α-s1casein, α-s2casein, β-casein and k-casein were determined by means of the same equipment and the same wavelength as above but the protocol was modified as follows: rough protein were reduced by incubation with 20 mM dithiothreitol for 1 h at 37°C then filtered through 0.45 µm membrane and finally acidified to pH 2 by dilution in a 0.1 % trifluoroacetic acid solution before injection in the high performance liquid chromatography (HPLC) and elution by a 30 to 46% acetonitrile gradient for 30 min at 40°C (Jaubert and Martin, 1992).

Lactose quantification

Lactose was determined on clarified supernatants obtained after deproteinization of samples by 3% sulfosalicylic acid for 1 h at 4°C, followed by centrifugation and filtration of the supernatants on 0.45 µm membranes. These filtrates were then diluted in ultra pure water (MilliQ, Millipore) to adjust the concentration of lactose to the standard curve. The analysis was carried out by ionic chromatography in a Dionex ICS 3000 HPLC system (Thermo Fisher S.A., Voisins le Bretonneux, France) by using of a Dionex Carbopac PA1, (4 x 250 mm) column. Elution was driven by a 12 to 200 mM sodium hydroxyde gradient for 22 min at 30°C. Lactose content was finally quantified by pulsed amperometric detection.

Mineral contents

Mineral content (calcium, magnesium, sodium, potassium, iron, copper, zinc, and manganese) were determined by atomic absorption spectrometer (Varian 220FS Spectr AA, Les Ulis, France) (Brulé et al., 1974). Phosphorus was determined by colorimetry according to IDF (1990).

Statistical analysis

Data on the compositions of milk and yogurt samples were evaluated by means of one-way analysis of variance using statistical package SPSS 16.0. Differences between samles were tested according to Tukey test and considered to be significant when P<0.05.

RESULTS AND DISCUSSION

Raw cow milks

pH and proximate composition of raw cow milk

Table 1 shows the pH and proximate composition of raw cow milk (dry matter, ash, fat, lactose, total protein, NPN, NCN, true protein, whey protein and casein). The mean pH of the milk was 6.25. This value was lower compared with the pH value of fresh raw cow milk (6.6-6.8) reported by Alais (1984). It was also lower compared to the value of 6.76 found by Ahmad et al. (2008) in raw cow milk (Holstein breed of *Bos taurus*) of France. The low value of milk pH obtained may be due to infection of the udder of the animal (Morgan, 1999). The mean dry matter content was 13.06 g/100 g. It was similar to 13.0 g/100 g reported by Mapekula et al. (2011) in milk from local crossbred cows in South Africa.

The mean ash content was 0.63 g/100 g. This

value was close to 0.65 g/100g found by Sanz Ceballos et al. (2009) in Holstein Friesian cow milk of south eastern Spain, but lower than 0.79 found by Bonfoh et al. (2005) in raw milk composition of Malian zebu cows (*Bos indicus*).

The mean fat was 4.48 g/100 g. This value was higher than 3.42 and 3.25 g/100 g found by Sanz Ceballos et al. (2009) in Holstein Friesian cow milk and Mapekula et al. (2011) in milk from local Nguni cows in South Africa, respectively. When milk has higher level fat, then it should be used to produce butter. The mean lactose content was 4.31 g/100 g. This value was close to 4.47/100g found by Sanz Ceballos et al. (2009) in Holstein Friesian cow milk. The total protein content ranged from 2.64 (M7) and 4.26 g/100 g (M3) with a mean of 3.26 g/100 g. This value was close to 3.30/100 g found by Mirzadeh et al. (2010) in Holstein cow milk of Lordegan Region of Iran. The protein content of milk is an essential feature of its market value since higher protein content enhances performance of technological transformation. Milk with high protein content is good for cheese production. These milk samples therefore represent a good raw material for the dairy industry. The means NPN and NCN were 0.21 and 0.87 g/100 g, respectively. These values were higher than that found by Ahmad et al. (2008) in raw cow milk (0.09 and 0.74 g/100 g) for NPN and NCN, respectively.

The lowest content of true protein (2.43 g/100 g) was found in (M7) while the highest (4.03 g/100 g) was found in (M3) with a mean of 3.05 g /100 g. This value was compared to 3.03/100 g found by Mayer et al. (2012) in cow milk of Austria. The whey protein content ranged from 0.51 g/100 g (M10) to 0.88 g /100 g (M6) with a mean of 0.66 g/100 g. This value was compared to 0.68 g/100g found by Czerniewcz et al. (2006) in Holstein-Friesian cows of Warmia in Olsztyn. The mean casein was 2.40 g/ 100 g. This value was closer to 2.61/100 g found by Ahmad et al. (2008) in raw cow milk. The physiological differences of the animal, stage of lactation and some common factors such as season, feed, breed, time and sequence of milking could be responsible for the differences observed in the composition of cow milk (Iggman et al., 2003; Farah et al., 2006; Slots et al., 2009; Mapekula et al., 2011; Frelich et al. 2012; Myburgh et al., 2012).

Amino acid composition of raw cow milk

The amino acid composition of the 11 cow milk samples is shown in Table 2. M7, with low protein content, had the lowest level of all amino acids except alanine, cysteine and methionine; while M9 had the highest level of all amino acid except glycine, cysteine and methionine. These values of amino acid were higher than those reported earlier by Mapekula et al. (2011) in milk from local crossbred cows in South Africa. The mean values of aspartic acid (2.42 g/kg), threonine (1.45 g/kg), serine (1.67 g/kg), alanine (1.06 g/kg), cysteine (0.21 g/kg), methionine (0.66 g/kg), isoleusine (1.57 g/kg), leucine (2.95 g/kg), tyrosine (1.53 g/kg), phenylalanine (1.50 g/kg), lysine (2.57 g/kg) and arginine (1.04 g/kg) were compared to the values found by Sanz Ceballos et al. (2009) in Holstein Friesian cows of south eastern Spain.

Protein fraction composition of raw cow milk

Table 3 shows the protein fraction composition of raw cow milk (α-lactalbumin, β-lactoglobulin, αS1-casein, αS2-casein, β-casein and κ-casein). The content of α-lactalbumin was between 0.62 (M2) and 1.10 g/kg (M6) with a mean of 0.84 g/kg. This value was similar to the value of 0.82 g/kg found by Mackle et al. (1999) in Friesian cows of New Zealand, but lower than 1.05 g/kg found by Barłowska et al. (2012) in cow milk. For the β-lactoglobulin content, the lowest value was found in M11 (3.21 g/kg) while the highest was in M8 (4.87 g/kg). The mean was 3.92 g/kg. This value was closer to 3.25 found by Barłowska et al. (2012) in cow milk. The content of αS1-casein ranged from 1.00 (M8) to 8.35 g/kg (M2) with a mean of 4.08 g/kg. The content of αS2-casein varied from 0.09 (M8) to 0.86 g/kg (M9) with a mean of 0.39 g/kg. The content of β-casein varied from 1.87 (M5) to 6.05 g/kg (M4) with a mean of 3.86 g/kg. The content of κ-casein was found in relatively low amount in M6 (1.02 g/kg) while high amount was found in M4 (2.76 g/kg). The β-lactoglobulin, αS1-casein + αS2-casein, β-casein and κ-casein means (3.92, 4.47, 3.86 and 1.98 g/kg), respectively, were lower than the values reported by Mackle et al. (1999) in Friesian cows of New Zealand (4.7, 12.70, 11.00 and 2.9 g/kg), respectively, for β-lactoglobulin, αS1-casein + αS2-casein, β-casein and κ-casein. The variation in milk samples and milk yield within a species depends on so many factors. Some of these factors are genetics, stage of lactation, daily variation, parity, type of diet, age, udder health and season (Iggman et al., 2003; Farah et al., 2006; Slots et al., 2009; Mapekula et al., 2011; Frelich et al. 2012; Myburgh et al., 2012).

Mineral composition of raw cow milk

Table 4 shows the content of Ca, Mg, Na, K, P, Fe, Zn, Cu and Mn in raw cow milk. Ca and Mg means were 1217 and 103 mg/kg, respectively. These values were higher than the corresponding values of 1135 and 94 mg/kg reported by Sanz Ceballos et al. (2009), in Holstein Friesian cows of South Eastern Spain. For the Na and K content, the means were 310 and 1384 mg/kg, respectively. These values were lower than the respective values of 430 and 1500 mg/kg reported by Sboui et al. (2009) for South Tunisian cows. P, Fe, Zn and Cu P means were 776, 0.72, 3.34 and 0.04 mg/kg, respectively. These values were lower than the corresponding values of 870, 0.9, 4.63 and 0.14 mg/kg found by Sanz Ceballos et al. (2009) in Holstein Friesian cows of South Eastern Spain. Mn was found in small amounts in the 11 milk samples.

Table 2. Amino acid composition of raw cow milk (g/Kg) collected in Maroua (Cameroon).

Sample	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	Mean
Asp	2.51±0.00^{c}	2.24±0.01^{e}	2.56±0.03^{bc}	2.63±0.00^{ab}	2.47±0.01^{c}	2.66±0.07^{a}	2.02±0.06^{f}	2.33±0.00^{d}	2.71±0.02^{a}	2.08±0.01^{f}	2.47±0.03^{c}	2.42±0.22
Thr	1.41±0.01^{cd}	1.29±0.01^{e}	1.50±0.02^{bc}	1.56±0.01^{b}	1.42±0.01^{cd}	1.56±0.09^{b}	1.16±0.03^{e}	1.45±0.01^{cd}	1.74±0.00^{a}	1.38±0.01^{de}	1.49±0.03^{bc}	1.45±0.14
Ser	1.72±0.01^{bc}	1.59±0.00^{e}	1.77±0.03^{b}	1.87±0.01^{a}	1.74±0.01^{bc}	1.65±0.08^{d}	1.39±0.04^{g}	1.64±0.01^{de}	1.91±0.00^{a}	1.46±0.01^{f}	1.70±0.00^{c}	1.67±0.15
Glu	6.16±0.02^{bcd}	5.68±0.00^{f}	6.30±0.06^{bc}	6.7±0.03^{a}	6.36±0.05^{b}	5.79±0.28^{f}	5.08±0.14^{g}	5.98±0.17^{cde}	6.87±0.00^{a}	5.23±0.00^{g}	5.96±0.03^{de}	6.01±0.54
Gly	0.65±0.02^{de}	0.60±0.00^{f}	0.65±0.01^{de}	0.69±0.00^{bc}	0.64±0.01^{e}	0.75±0.02^{a}	0.55±0.02^{g}	0.63±0.01^{ef}	0.71±0.00^{b}	0.55±0.00^{g}	0.67±0.01^{cd}	0.64±0.06
Ala	1.05±0.02^{c}	0.94±0.00^{d}	1.14±0.01^{a}	1.14±0.01^{a}	1.06±0.01^{c}	1.16±0.03^{a}	0.92±0.03^{d}	1.11±0.00^{ab}	1.16±0.00^{a}	0.89±0.01^{d}	1.07±0.01^{bc}	1.06±0.095
Val	2.03±0.02^{c}	1.87±0.00^{de}	2.05±0.02^{c}	2.23±0.03^{b}	2.06±0.01^{c}	1.93±0.05^{d}	1.74±0.07^{f}	1.93±0.00^{d}	2.32±0.00^{a}	1.84±0.01^{e}	2.08±0.01^{c}	2.01±0.16
Cys	0.17±0.02^{d}	0.17±0.00^{d}	0.23±0.00^{ab}	0.23±0.02^{ab}	0.19±0.00^{bcd}	0.27±0.02^{a}	0.19±0.01^{bcd}	0.22±0.03^{bc}	0.23±0.02^{ab}	0.18±0.00^{cd}	0.21±0.00^{bcd}	0.21±0.03
Met	0.85±0.00^{a}	0.75±0.02^{bc}	0.83±0.01^{ab}	0.91±0.03^{a}	0.84±0.00^{a}	0.46±0.01^{e}	0.66±0.02^{c}	0.50±0.03^{de}	0.56±0.00^{d}	0.36±0.05^{f}	0.52±0.08^{de}	0.66±0.18
Ile	1.65±0.02^{bcd}	1.39±0.00^{e}	1.64±0.02^{bcd}	1.73±0.03^{ab}	1.62±0.01^{bcd}	1.54±0.11^{d}	1.34±0.04^{e}	1.56±0.00^{cd}	1.82±0.00^{a}	1.36±0.01^{e}	1.67±0.01^{bc}	1.57±0.15
Leu	3.01±0.01^{c}	2.76±0.01^{e}	3.05±0.02^{c}	3.24±0.02^{b}	3.07±0.03^{c}	3.07±0.03^{c}	2.64±0.07^{f}	2.92±0.02^{d}	3.39±0.01^{a}	2.67±0.02^{ef}	3.05±0.04^{c}	2.95±0.23
Tyr	1.58±0.01^{cd}	1.38±0.01^{e}	1.63±0.01^{bc}	1.71±0.02^{ab}	1.60±0.02^{cd}	1.40±0.07^{e}	1.28±0.06^{f}	1.53±0.01^{d}	1.79±0.00^{a}	1.30±0.01^{e}	1.70±0.02^{b}	1.53±0.17
Phe	1.55±0.02^{b}	1.39±0.01^{c}	1.55±0.02^{b}	1.69±0.01^{a}	1.56±0.01^{b}	1.40±0.07^{c}	1.29±0.05^{d}	1.43±0.01^{c}	1.73±0.00^{a}	1.35±0.01^{cd}	1.59±0.02^{b}	1.50±0.13
Lys	2.60±0.01^{cd}	2.34±0.02^{e}	2.70±0.03^{bc}	2.78±0.02^{b}	2.67±0.02^{c}	2.65±0.09^{c}	2.18±0.07^{f}	2.53±0.02^{d}	2.94±0.00^{a}	2.24±0.02^{ef}	2.69±0.01^{bc}	2.57±0.22
His	0.82±0.00^{bc}	0.74±0.01^{de}	0.83±0.01^{bc}	0.90±0.01^{a}	0.85±0.01^{b}	0.80±0.03^{c}	0.68±0.03^{f}	0.76±0.00^{d}	0.93±0.00^{a}	0.72±0.01^{ef}	0.85±0.00^{b}	0.81±0.07
Arg	1.10±0.03^{b}	1.00±0.00^{c}	1.10±0.00^{b}	1.10±0.00^{b}	1.07±0.058^{bc}	1.03±0.58^{bc}	0.87±0.058^{d}	1.00±0.00^{c}	1.20±0.00^{a}	0.90±0.01^{d}	1.10±0.01^{b}	1.04±0.09
Pro	3.11±0.00^{b}	2.78±0.00^{c}	3.11±0.07^{b}	3.48±0.02^{a}	3.14±0.03^{b}	2.81±0.11^{c}	2.62±0.06^{d}	2.87±0.02^{c}	3.60±0.00^{a}	2.79±0.07^{c}	3.20±0.01^{b}	3.04±0.29

Mean values in the same row with different superscript letters are significantly different ($P < 0.05$).

Table 3. Protein fraction composition of raw cow milk (g/kg) collected in Maroua (Cameroon).

Sample	α-Lactalbumin	β-Lactoglobulin	αS1-casein	αS2-casein	β-casein	κ-casein
M1	1.04±0.01^{b}	4.12±0.25^{cd}	7.47±0.18^{a}	0.70±0.03^{b}	4.63±0.08^{b}	2.72±0.01^{a}
M2	0.62±0.01^{h}	3.88±0.01^{de}	8.35±0.46^{a}	0.52±0.02^{c}	5.09±0.16^{b}	2.24±0.00^{b}
M3	0.65±0.01^{g}	4.62±0.00^{ab}	5.25±0.25^{b}	0.15±0.04^{e}	2.68±0.05^{cd}	1.75±0.07^{c}
M4	0.93±0.01^{c}	3.54±0.05^{ef}	7.54±0.74^{a}	0.44±0.07^{cd}	6.05±0.40^{a}	2.76±0.25^{a}
M5	0.85±0.01^{d}	3.35±0.03^{f}	2.97±0.36^{c}	0.16±0.01^{e}	1.87±0.14^{d}	1.17±0.18^{ef}
M6	1.10±0.00^{a}	4.60±0.29^{ab}	1.31±0.51^{de}	0.46±0.07^{cd}	2.57±0.13^{cd}	1.02±0.02^{f}
M7	0.85±0.01^{d}	4.29±0.04^{bc}	1.75±0.02^{de}	0.14±0.01^{e}	2.98±0.11^{c}	1.21±0.30^{def}
M8	0.85±0.01^{d}	4.87±0.04^{a}	1.00±0.08^{e}	0.09±0.02^{e}	3.00±0.17^{c}	1.61±0.03^{cde}
M9	0.85±0.02^{d}	3.42±0.09^{f}	5.88±0.10^{b}	0.86±0.03^{a}	5.15±0.06^{b}	3.04±0.00^{a}
M10	0.78±0.00^{e}	3.22±0.00^{f}	1.19±0.04^{de}	0.41±0.01^{d}	3.45±0.35^{c}	1.66±0.11^{cd}
M11	0.70±0.00^{f}	3.21±0.00^{f}	2.23±0.40^{cd}	0.36±0.01^{d}	5.05±0.75^{b}	2.64±0.27^{ab}
Mean	0.84±0.14	3.92±0.6	4 .08±2.78	0.39±0.24	3.86±1.34	1.98±0.71

Mean values in the same column with different superscript letters are significantly different ($P < 0.05$).

Table 4. Mineral composition of raw cow milk (mg/Kg) collected in Maroua (Cameroon).

Sample	Ca	Mg	Na	K	P	Fe	Zn	Cu	Mn
M1	1228±4.78^{e}	107±0.05^{b}	361±1.52^{b}	1561±1.95^{b}	814±2.84^{c}	0.87±0.00^{a}	2.30±0.34^{e}	0.04±0.00^{bc}	0.02±0.00^{b}
M2	1301±6.42^{b}	113±0.20^{ab}	313±0.84^{e}	1680±5.84^{a}	811±5.12^{c}	0.66±0.08^{d}	1.03±0.02^{f}	0.03±0.00^{c}	0.03±0.00^{ab}
M3	1246±7.65^{d}	119±5.62^{a}	295±1.86^{fg}	1555±8.50^{b}	864±0.00^{b}	0.67±0.03^{cd}	3.32±0.24^{d}	0.03±0.00^{cd}	0.05±0.02^{a}
M4	1151±7.50^{g}	94±1.21^{cd}	298 ±3.45^{f}	1385±2.16^{d}	711±16.65^{ef}	0.80±0.07^{abc}	4.70±0.17^{b}	0.06±0.00^{a}	0.02±0.01^{b}
M5	1120±7.67^{h}	87±1.72^{de}	289±0.78^{g}	1367±4.44^{e}	870±37.04^{b}	0.64±0.01^{d}	3.94±0.16^{cd}	0.04±0.00^{bc}	0.02±0.00^{b}
M6	1551±3.56^{a}	114±0.59^{ab}	384±0.86^{a}	1428±5.99^{c}	998±8.31^{a}	0.65±0.03^{d}	6.21±0.16^{a}	0.05±0.00^{a}	0.02±0.00^{b}
M7	963±5.38^{i}	79±0.34^{f}	242±3.67^{i}	1212±1.76^{g}	626±1.92^{i}	0.64±0.03^{d}	1.37±0.35^{f}	0.02±0.00^{e}	0.01±0.00^{b}
M8	1238±1.52^{de}	99±4.70^{c}	301±4.34^{f}	1269±4.00^{f}	738±27.27^{de}	0.85±0.08^{ab}	4.21±0.44^{bc}	0.03±0.00^{cd}	0.01±0.00^{b}
M9	1276±6.20^{c}	117±0.42^{a}	334±0.83^{c}	1376±3.89^{de}	783±12.87^{cd}	0.64±0.02^{d}	3.24±0.32^{d}	0.04±0.00^{bc}	0.02±0.00^{b}
M10	1186±1.54^{f}	118±0.40^{a}	322±0.49^{d}	1282±9.83^{f}	681±0.00^{gh}	0.72±0.00^{bcd}	3.95±0.10^{cd}	0.04±0.00^{bc}	0.02±0.00^{b}
M11	1127±6.30^{h}	82±0.30^{ef}	267±2.33^{h}	1104±4.73^{h}	636±0.00^{hi}	0.74±0.07^{abcd}	2.41±0.12^{e}	0.02±0.00^{e}	0.03±0.00^{ab}
Mean	1217±140.67	103±14.63	310±39.00	1384±162.65	776±109.37	0.72±0.094	3.34±1.47	0.04±0.01	0.02±0.01

Mean values in the same column with different superscript letters are significantly different (P< 0.05).

The mean was 0.02 mg/kg. This value was also lower than 0.056 mg/kg found by Enb et al. (2009) in cow milk from Egypt. The physiological differences in the animals, stage of lactation and factors such as season, feed, breed, time and sequence of milking could be responsible for the differences observed in the composition of the cow milk (Iggman et al., 2003; Farah et al., 2006; Slots et al., 2009; Mapekula et al., 2011; Frelich et al. 2012; Myburgh et al., 2012).

Artisanal yoghurt

pH and proximate composition of artisanal yoghurt

Table 5 shows the pH and proximate composition of artisanal yoghurt (dry matter, ash, fat, lactose, total protein, NPN, NCN, true protein, whey protein and casein). The mean pH of yoghurt was 3.84. This value was lower than 4.3 reported by Abdulrahman et al. (1998) in fermented dairy products consumed in Bahrain. The mean dry matter content was 20.11 g /100 g. This value was higher than 10.40 g/100 g found by Enb et al. (2009) in artisanal yoghurt from cow milk of Egypt. The lowest ash content was found in YD while the highest was found in YL with a mean value of 0.61 g /100 g. The fat content was between 1.25 (YD) and 4.05 g/100 g (YH) with a mean of 2.66 g/100 g. YE had the lowest lactose content while the highest was found in YK. The mean was 2.41 g /100 g. The total protein content ranged from 2.13 (YC) and 3.63 g /100 g (YL) with a mean of 2.83 g/100 g. The mean values for ash, fat, lactose and total protein were lower than those found by Abdulrahman et al. (1998) in fermented dairy products consumed in Bahrain. The mean values of NPN and NCN were 0.21 and 0.36 g /100 g, respectively. The lowest content of true protein was found in YC while the highest was found in YL. The mean was 2.62 g /100 g. The whey protein content ranged from 0.02 (YB) to 0.41 g/100 g (YG) with a mean of 0.14 g /100 g. The casein content varied from 1.89 (YE) to 3.23 g/100 g (YL) with a mean of 2.48 g/100 g. These varia-tions in chemical composition between artisanal yoghurts can be attributed to several factors such as type of milk used, method of preparation, type and proportion of ingredients used.

Amino acid composition of artisanal yoghurt

Table 6 presents the amino acid composition of artisanal yoghurt. YL, with high protein content had the highest level of all amino acid. The main determinant of food protein quality is the content and availability of essential amino acids (Robbins et al., 2010). These nutrients have been shown to play an important role in the growth, reproduction and maintenance of the human body (FAO/WHO/UNU, 2007).

Protein fraction composition of artisanal yoghurt

Table 7 shows the protein fraction composition of artisanal yoghurt. The mean values of α-lactalbumin,

Table 5. pH and proximate composition of artisanal yoghurt (g/100g) collected in Maroua (Cameroon).

Sample	pH	Dry matter	Ash	Fat	Lactose	Total protein	NPN	NCN	True protein	Whey protein	Casein
YA	3.95± 0.00^{c}	20.26±0.05^{f}	0.61±0.01^{cd}	2.30±0.00^{g}	2.71±0.01^{c}	2.75±0.00^{g}	0.22±0.00^{d}	0.27±0.01^{h}	2.53±0.00^{f}	0.05±0.01^{ef}	2.48±0.00^{f}
YB	3.61± 0.00^{j}	23.03±0.03^{a}	0.67±0.00^{b}	2.95±0.05^{d}	3.05±0.11^{b}	2.90±0.00^{e}	0.29±0.00^{a}	0.31±0.00^{g}	2.61±0.00^{e}	0.02±0.00^{g}	2.59±0.00^{e}
YC	3.73± 0.00^{g}	17.27±0.02^{i}	0.51±0.01^{fg}	2.40±0.00^{fg}	1.88±0.02^{ef}	2.13±0.00^{k}	0.19±0.00^{g}	0.33±0.01^{fg}	1.94±0.00^{i}	0.14±0.01^{d}	1.80±0.00^{j}
YD	3.73± 0.00^{g}	13.00±0.05^{j}	0.46±0.01^{g}	1.25±0.05^{j}	1.62±0.02^{gh}	2.29±0.01^{i}	0.17±0.00^{h}	0.36±0.00^{e}	2.12±0.01^{h}	0.19±0.00^{c}	1.93±0.01^{i}
YE	3.8± 0.00^{e}	23.04±0.01^{a}	0.50±0.00^{g}	2.05±0.05^{h}	1.54±0.01^{h}	2.23±0.00^{j}	0.16±0.00^{i}	0.34±0.00^{ef}	2.07±0.00^{h}	0.18±0.00^{c}	1.89±0.00^{i}
YF	3.78± 0.00^{f}	22.42±0.04^{b}	0.64±0.00^{bc}	2.45±0.05^{f}	2.66±0.03^{c}	3.24±0.01^{b}	0.21±0.00^{e}	0.25±0.00^{h}	3.03±0.02^{b}	0.04±0.00^{efg}	2.99±0.01^{b}
YG	3.88± 0.00^{d}	19.51±0.06^{g}	0.65±0.01^{bc}	3.60±0.00^{b}	2.92±0.07^{b}	3.21±0.04^{b}	0.23±0.01^{d}	0.63±0.03^{a}	2.98±0.04^{bc}	0.41±0.02^{a}	2.61±0.04^{e}
YH	3.67± 0.00^{i}	21.71±0.13^{d}	0.58±0.05^{de}	4.05±0.05^{a}	2.27±0.03^{d}	3.07±0.04^{d}	0.25±0.01^{c}	0.27±0.00^{h}	2.82±0.04^{d}	0.03±0.01^{fg}	2.80±0.04^{d}
YI	3.69± 0.00^{h}	20.46±0.02^{e}	0.61±0.02^{cd}	2.75±0.05^{e}	1.76±0.02^{fg}	2.84±0.00^{f}	0.20±0.00^{f}	0.43±0.00^{c}	2.64±0.00^{e}	0.23±0.00^{b}	2.41±0.00^{g}
YJ	3.79± 0.00^{ef}	18.93±0.01^{h}	0.55±0.00^{ef}	1.90±0.00^{i}	1.92±0.02^{e}	2.58±0.00^{h}	0.22±0.00^{d}	0.46±0.00^{b}	2.36±0.00^{g}	0.24±0.00^{b}	2.12±0.00^{h}
YK	4.12± 0.00^{b}	19.59±0.09^{g}	0.69±0.00^{b}	2.75±0.05^{e}	4.04±0.05^{a}	3.12±0.00^{c}	0.16±0.00^{i}	0.22±0.00^{i}	2.96±0.00^{c}	0.06±0.00^{e}	2.90±0.00^{c}
YL	4.30± 0.00^{a}	22.07±0.01^{c}	0.81±0.02^{a}	3.45±0.05^{c}	2.61±0.09^{c}	3.63±0.00^{a}	0.28±0.00^{b}	0.40±0.00^{d}	3.35±0.01^{a}	0.12±0.00^{d}	3.23±0.00^{a}
Mean	3.84±0.19	20.11±2.77	0.61±0.09	2.66±0.76	2.41±0.71	2.83±0.44	0.21±0.04	0.36±0.11	2.62±0.42	0.14±0.11	2.48±0.45

NCN, Non-casein nitrogen; NPN, non-protein nitrogen. Mean values in the same column with different superscript letters are significantly different (P< 0.05).

Table 6. Amino acid composition of artisanal yoghurt (g/Kg) collected in Maroua (Cameroon).

Sample	YA	YB	YC	YD	YE	YF	YG	YH	YI	YJ	YK	YL	Mean
Asp	2.03±0.02^{e}	2.18±0.03^{d}	1.60±0.00^{g}	1.66±0.01^{g}	1.60±0.00^{g}	2.30±0.00^{c}	2.38±0.05^{b}	2.21±0.04^{d}	2.07±0.02^{e}	1.84±0.00^{f}	2.35±0.00^{bc}	2.64±0.01^{a}	2.07±0.32
Thr	1.32±0.01^{f}	1.40±0.03^{e}	1.02±0.02^{j}	1.08±0.00^{h}	1.03±0.00^{i}	1.50±0.00^{c}	1.59±0.04^{b}	1.44±0.01^{d}	1.30±0.01^{f}	1.21±0.02^{g}	1.57±0.01^{b}	1.71±0.00^{a}	1.35±0.22
Ser	1.35±0.01^{d}	1.39±0.01^{cd}	1.04±0.02^{f}	1.12±0.00^{ef}	1.07±0.01^{f}	1.57±0.00^{b}	1.62±0.07^{b}	1.45±0.02^{c}	1.33±0.02^{d}	1.18±0.01^{e}	1.63±0.00^{b}	1.72±0.01^{a}	1.37±0.22
Glu	5.31±0.01^{d}	5.51±0.02^{c}	4.12±0.02^{h}	4.41±0.02^{f}	4.31±0.00^{fg}	6.18±0.00^{b}	6.18±0.20^{b}	5.63±0.09^{c}	5.29±0.05^{d}	4.61±0.01^{e}	6.24±0.01^{b}	6.63±0.00^{a}	5.37±0.81
Gly	0.49±0.00^{e}	0.56±0.01^{d}	0.38±0.00^{h}	0.42±0.00^{fg}	0.40±0.00^{gh}	0.62±0.01^{b}	0.61±0.01^{b}	0.58±0.02^{c}	0.51±0.01^{e}	0.44±0.01^{f}	0.63±0.01^{b}	0.65±0.00^{a}	0.52±0.09
Ala	0.88±0.01^{e}	1.04±0.00^{bc}	0.71±0.00^{g}	0.74±0.00^{g}	0.71±0.01^{g}	1.01±0.01^{cd}	1.05±0.02^{b}	1.00±0.03^{d}	0.91±0.01^{e}	0.81±0.01^{f}	1.06±0.01^{b}	1.15±0.01^{a}	0.92±0.14
Val	1.60±0.02^{e}	1.73±0.01^{d}	1.26±0.00^{h}	1.33±0.01^{g}	1.31±0.00^{g}	1.93±0.00^{b}	1.94±0.04^{b}	1.80±0.04^{c}	1.63±.00^{e}	1.41±0.01^{f}	1.92±0.00^{b}	2.10±0.00^{a}	1.66±0.27
Cys	0.08±0.00^{bc}	0.07±0.00^{bc}	0.06±0.00^{c}	0.07±0.00^{bc}	0.05±0.00^{c}	0.05±0.00^{c}	0.12±0.06^{ab}	0.13±0.02^{ab}	0.12±0.03^{ab}	0.11±0.00^{bc}	0.09±0.00^{bc}	0.17±0.00^{a}	0.09±0.04
Met	0.11±0.00^{cd}	0.00±0.00^{h}	0.03±0.00^{g}	0.05±0.00^{ef}	0.05±0.00^{ef}	0.15±0.00^{b}	0.08±0.01^{d}	0.09±0.02^{d}	0.10±0.00^{cd}	0.05±0.00^{ef}	0.12±0.00^{c}	0.21±0.00^{a}	0.09±0.06
Ile	1.27±0.05^{de}	1.30±0.00^{cd}	1.00±0.00^{g}	1.03±0.05^{fg}	1.00±0.00^{g}	1.50±0.00^{b}	1.47±0.05^{b}	1.37±0.05^{c}	1.20±0.00^{e}	1.10±0.00^{f}	1.50±0.00^{b}	1.60±0.00^{a}	1.28±0.21
Leu	2.45±0.01^{d}	2.61±0.04^{c}	1.93±0.01^{g}	2.00±0.00^{f}	2.00±0.00^{f}	2.91±0.00^{b}	2.96±0.05^{b}	2.75±0.05^{c}	2.50±0.00^{d}	2.12±0.00^{e}	2.92±0.01^{b}	3.19±0.00^{a}	2.53±0.41
Tyr	1.13±0.01^{bc}	1.18±0.03^{b}	0.88±0.00^{de}	0.99±0.00^{c}	0.84±0.00^{e}	1.02±0.00^{e}	1.19±0.02^{b}	1.13±0.02^{bc}	1.02±0.12^{c}	0.96±0.00^{cd}	1.13±0.00^{bc}	1.40±0.00^{a}	1.07±0.15
Phe	1.21±0.01^{e}	1.26±0.03^{d}	0.94±0.00^{h}	1.00±0.00^{g}	0.96±0.00^{gh}	1.45±0.00^{b}	1.47±0.03^{b}	1.37±0.03^{c}	1.23±0.01^{de}	1.06±0.00^{f}	1.46±0.00^{b}	1.60±0.00^{a}	1.25±0.21
Lys	2.11±0.00^{f}	2.25±0.01^{e}	1.67±0.00^{i}	1.75±0.01^{h}	1.70±0.00^{hi}	2.48±0.00^{c}	2.54±0.00^{b}	2.31±0.04^{d}	2.15±0.02^{f}	1.88±0.00^{g}	2.52±0.00^{bc}	2.70±0.00^{a}	2.17±0.34
His	0.64±0.01^{e}	0.69±0.00^{d}	0.50±0.00^{h}	0.55±0.00^{g}	0.51±0.00^{h}	0.77±0.00^{b}	0.78±0.02^{b}	0.73±0.02^{c}	0.67±0.01^{d}	0.58±0.00^{f}	0.78±0.00^{b}	0.89±0.00^{a}	0.67±0.12
Arg	0.86±0.01^{e}	0.89±0.01^{d}	0.67±0.01^{h}	0.72±0.01^{g}	0.71±0.03^{g}	0.93±0.00^{c}	0.99±0.01^{b}	0.92±0.01^{c}	0.85±0.01^{e}	0.76±0.00^{f}	1.00±0.00^{b}	1.11±0.01^{a}	0.87±0.13
Pro	2.44±0.00^{e}	2.66±0.03^{d}	1.93±0.02^{h}	2.05±0.01^{g}	2.00±0.01^{gh}	3.00±0.00^{b}	3.01±0.11^{b}	2.80±0.10^{c}	2.48±0.02^{e}	2.15±0.00^{f}	2.95±0.05^{b}	3.16±0.03^{a}	2.55±0.42

Mean values in the same row with different superscript letters are significantly different (P < 0.05).

Table 7. Protein fraction composition of artisanal yoghourt (g/kg) collected in Maroua (Cameroon).

Sample	α-Lactalbumin	β-Lactoglobulin	αS1-casein	αS2-casein	β-casein	κ-casein
YA	0.00±0.00^{h}	0.42±0.02^{f}	4.63±0.16^{b}	0.15±0.02^{d}	5.63±0.37^{d}	0.00±0.00^{d}
YB	0.00±0.00^{h}	0.00±0.00^{g}	5.20±0.09^{a}	0.21±0.00^{bc}	6.41±0.10^{bc}	0.00±0.00^{d}
YC	0.24±0.00^{e}	0.83±0.03^{e}	2.01±0.06^{e}	0.09±0.00^{e}	1.69±0.04^{h}	0.00±0.00^{d}
YD	0.35±0.00^{d}	1.30±0.02^{d}	3.25±0.27^{c}	0.17±0.01^{cd}	2.83±0.22^{g}	0.00±0.00^{d}
YE	0.35±0.01^{d}	1.33±0.03^{d}	4.56±0.04^{b}	0.28±0.01^{a}	4.43±0.06^{e}	0.00±0.00^{d}
YF	0.20±0.00^{f}	0.41±0.01^{f}	5.05±0.06^{ab}	0.17±0.01^{cd}	6.97±0.02^{b}	0.22±0.01^{c}
YG	1.01±0.01^{a}	3.68±0.03^{a}	2.34±0.24^{de}	0.13±0.03^{de}	3.52±0.18^{f}	0.89±0.04^{b}
YH	0.00±0.00^{h}	0.43±0.03^{f}	1.83±0.05^{e}	0.17±0.04^{cd}	6.05±0.07^{cd}	0.85±0.15^{b}
YI	0.58±0.01^{b}	1.86±0.02^{b}	5.44±0.19^{a}	0.22±0.01^{b}	5.14±0.02^{d}	0.00±0.00^{d}
YJ	0.59±0.02^{b}	1.71±0.02^{c}	4.62±0.31^{b}	0.15±0.00^{d}	4.15±0.26^{e}	0.00±0.00^{d}
YK	0.09±0.00^{g}	0.41±0.00^{f}	2.71±0.08^{d}	0.20±0.00^{bc}	5.66±0.10^{d}	0.00±0.00^{d}
YL	0.44±0.01^{c}	0.43±0.02^{f}	2.78±0.28^{cd}	0.15±0.02^{d}	7.74±0.35^{a}	1.65±0.08^{a}
Mean	0.32±0.29	1.07±0.98	3.70±1.31	0.17±0.05	5.02±1.72	0.30±0.52

Mean values in the same column with different superscript letters are significantly different ($P < 0.05$).

Table 8. Mineral composition of artisanal yoghurt (mg/Kg) collected in Maroua (Cameroon).

Sample	Ca	Mg	Na	K	P	Fe	Zn	Cu	Mn
YA	1005±5.12^{f}	96±0.32^{e}	294±0.76^{e}	1383±0.63^{f}	766±1.46^{d}	0.55±0.04^{b}	3.09±0.04^{cde}	0.14±0.05^{ab}	0.00±0.00^{a}
YB	1129±0.37^{d}	109±1.34^{d}	319±0.32^{c}	1528±2.31^{b}	815±5.12^{c}	0.51±0.01^{bc}	3.97±0.08^{b}	0.06±0.01^{bcd}	0.00±0.00^{a}
YC	719±3.65^{f}	81±0.24^{g}	282±1.60^{f}	1109±0.14^{k}	683±5.10^{f}	0.98±0.04^{a}	2.96±0.28^{de}	0.07±0.03^{bcd}	0.00±0.00^{a}
YD	697±3.01^{k}	74±0.45^{h}	207±0.03^{i}	991±3.83^{l}	635±3.69^{g}	0.37±0.04^{cd}	2.48±0.10^{f}	0.12±0.00^{abc}	0.00±0.00^{a}
YE	798±8.24^{i}	80±0.08^{g}	216±0.54^{h}	1140±3.64^{j}	639±3.37^{g}	0.52±0.10^{bc}	2.81±0.01^{ef}	0.04±0.01^{cd}	0.00±0.00^{a}
YF	1080±3.97^{e}	110±0.17^{d}	329±0.30^{b}	1406±5.01^{e}	755±0.00^{d}	0.50±0.08^{bc}	3.98±0.01^{b}	0.09±0.06^{abcd}	0.00±0.00^{a}
YG	1158±6.40^{c}	125±1.16^{b}	326±7.04^{bc}	1351±2.29^{g}	816±8.37^{c}	0.57±0.01^{b}	4.41±0.24^{a}	0.05±0.01^{bcd}	0.00±0.00^{a}
YH	1005±2.34^{f}	108±0.91^{d}	304±4.82^{d}	1207±7.27^{i}	714±6.07^{e}	0.54±0.02^{b}	3.45±0.04^{c}	0.02±0.00^{d}	0.00±0.00^{a}
YI	972±6.61^{g}	106±0.57^{e}	288±2.07^{ef}	1472±0.01^{d}	756±0.00^{d}	0.48±0.00^{bc}	3.31±0.07^{cd}	0.06±0.01^{bcd}	0.00±0.00^{a}
YJ	872±1.36^{h}	91±1.36^{f}	251±0.62^{g}	1253±7.20^{h}	713±4.36^{e}	0.24±0.04^{d}	3.03±0.07^{df}	0.05±0.02^{bcd}	0.00±0.00^{a}
YK	1249±7.53^{a}	119±0.77^{c}	350±0.19^{a}	1487±3.82^{c}	855±3.94^{b}	0.52±0.10^{bc}	4.44±0.01^{a}	0.05±0.03^{bcd}	0.00±0.00^{a}
YL	1209±0.64^{b}	135±0.50^{a}	350±0.97^{a}	1808±1.70^{a}	916±24.26^{a}	0.57±0.01^{b}	4.32±0.20^{ab}	0.16±0.07^{a}	0.00±0.00^{a}
Mean	991±180.98	103±18.33	293±49.39	1345±215.509	755±83.10	0.53±0.17	3.52±0.66	0.07±0.05	0.00±0.00

Mean values in the same column with different superscript letters are significantly different ($P< 0.05$).

β-lactoglobulin, αS1-casein, αS2-casein, β-Casein and κ-casein were 0.32, 1.07, 0.17, 3.70, 0.17, 5.02 and 0.30 g/kg). These variations in chemical composition between artisanal yoghurts samples can be attributed to several factors such as type of milk used, method of preparation, type and proportion of ingredients used.

Mineral composition of artisanal yoghurt

Table 8 shows the contents of Ca, Mg, Na, K, P, Fe, Zn, Cu and Mn in artisanal yoghurts. YL had the highest level of all these minerals except Ca and Fe. Mn was not detected in yoghurt samples. The means for Ca (991 mg/kg), Mg (103 mg/kg), Na (293 mg/kg), and P (755 mg/kg) were lower than those found by Abdulrahman et al. (1998) which reported respective values of (1670, 134, 750 and 1170 mg/kg) in fermented dairy products consumed in Bahrain. The means for Fe (0.53 mg/kg), and Zn (3.52 mg/kg) were higher com-pared with those found by Enb et al. (2009) which reported respective values of 0.49 and 2.63 mg/kg in artisanal yoghurt from cow milk from Egypt, but the mean for Cu (0.07 mg/kg) was lower than the value of 0.12 mg/kg found by Enb et al. (2009). These variations in mineral composition between traditional yoghurt samples can be attributed to several factors such as type of milk used, method of preparation, type and proportion of ingredients used.

Conclusion

The chemical composition of raw cow milk and artisanal yoghurt consumed in Maroua (Cameroon) varied from one sample to another. In general, some chemical of the values for the milk and yo-ghurt studied differed from those of previous stu-dies. This was particularly true for pH, NPN, pro-tein fraction (α- lactabumin, β-lactoglobulin, αS-casein, β-casein and κ-casein), some minerals and amino acids. Information on chemical compo-sition of milk and traditional yoghurt available in this study would be helpful for food scientists, nutritionists and public health workers interested in nutritive values of local foods. Further investigations on composition of other artisanal dairy products, such as cheese and dairy sweets, are highly recommended, in order to provide adequate data on food composition in Cameroon. Chemical determination should be expanded to include fatty acid and vitamins.

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Full Length Research Paper

Cloning of a vacuolar H^+-pryophosphatase gene from emphemeral plant *Olimarabidopsis pumila* whose overexpression improve salt tolerance in tobacco

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Olimarabidopsis pumila **is a close relative of the model plant** ***Arabidopsis thaliana*** **but, unlike** ***A. thaliana*****, it is a salt-tolerant ephemeral plant that is widely distributed in semi-arid and semi-salinized regions of the Xinjiang region of China, thus providing an ideal candidate plant system for salt tolerance gene mining. The vacuolar H^+-translocating inorganic pyrophosphatase (V-H^+-PPase) is an electrogenic proton pump that play pivotal role in translocating protons into vacuoles in plant cells. A V-H^+-PPase gene, *OpVP*, was isolated from *O. pumila* in this study. The *OpVP* cDNA has an open reading frame of 2 313 bp, encoding a polypeptide of 770 amino acid residues with an estimated molecular mass of 80.7 kDa. The OpVP shows high amino acid similarity with other Brassicaceae V-H^+-PPase genes. Expression profiles under salt and drought treatment, abcisic acid (ABA), indole-3-aceticacid (IAA) and gibberellins (GA) induction were investigated, and the results reveal that the expression of *OpVP* was induced in leaves under treatment with salt, drought, ABA, IAA and GA3. Overexpression of the *OpVP* gene confers enhanced salt tolerance to the transformed tobacco. Transgenic tobacco grows well in the presence of 200 mM NaCl, while wild-type plants exhibit chlorosis and growth inhibition, even death. Compared with wild-type, transgenic plants accumulated more Na^+ in leaves. Moreover, the leaves of transgenic plants retain higher chlorophyll content during salt stress. This study shows that *OpVP* is a potential gene for salt tolerance, and can be used in future for developing salt tolerant crops.**

Key words: H^+-pyrophoshatase, *Olimarabidopsis pumila*, salt, abiotic stress.

INTRODUCTION

Abiotic stress, such as drought, salinity and extreme temperature, is one of the primary causes of crop loss worldwide, reducing average production of major crop plants. Salinity is a major environmental factor limiting crop growth and productivity (Ashraf, 1994). It was estimated that 20% of all cultivated land, and nearly half of irrigated land, is affected by salinity (Rhoades and Loveday, 1990). In China, more than 90 million hectares of the land are exposed to salinity or secondary salinity. Xinjiang occupies one-sixth of the area of China, but more than one-third of the land in Xinjiang is salinized. Therefore, developing salt-tolerant varieties of crops is an important breeding goal in Xinjiang. Owing to extreme ecological environment, however, salt tolerant organisms are abundant in Xinjiang. An important research project is to mine salt-resistance genes from such plants which can

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Abbreviation: PCR, polymerase chain reaction; **qRT-PCR,** quantitative Real-time RT-PCR; **RACE,** rapid amplification of cDNA ends; **dNTPs,** deoxyribonucleotide tri phosphate; **ABA,** abcisic acid; **IAA,** indo-3-aceticacid; **GA,** gibberellins; V-H^+-PPase,vacuolar H^+-pyrophosphatase; **PPi,** inorganic pyrophosphate; CS, conserved segment; **ORF,** open reading frame.

be used in future crop improvement.

Olimarabidopsis pumila (Stephan) Al-Shehbaz, O'Kane & R. A. Price [synonym: *Arabidopsis pumila* (Stephan) N. Busch] habitats semi-arid and semi-salinized land in Xinjiang. *O*. *pumila* is a close relative of *Arabidopsis thaliana*, but it is more tolerant to salt stress than *A*. *thaliana* (Hoffmann et al., 2010; Roy et al., 2010). Although the size of 1 C DNA (173 Mbp) of *O. pumila* is comparable to that of *A*. *thaliana* (167 Mbp), its genome size is about twice as large as that of *A*. *thaliana* (Hoffmann et al., 2010). *O*. *pumila* has 2n = 32 chromosomes, but it has the lowest mean DNA content per chromosome of the four Brassicaceae species *A. thaliana*, *Arabis auriculata*, and *Arabis montbretiana*. The estimated time of divergence of *A. thaliana* and *O. pumila* was 10 - 14 million years (Mya) (Clauss and Koch, 2006; Roy et al., 2010).

To cope with salt stress, plants have developed multifarious adaptation mechanisms to tolerate high concentrations of salt in the environment (Hamada et al., 2001). In high salt stress environment, plant can survive in salt stress environment by exclusion of excess Na^+ from the cytoplasm and sequestration of Na^+ from cytosol to vacuole towards the maintenance of ion homeostasis inside the cell. Many studies concentrated on the Na^+/H^+ antiporter proteins in the plasma membrane and tono-plast which play essential roles in Na^+ exclusion and compartmentalization, for example SOS1 (Qiu et al., 2003), NHX1 (Pardo et al., 2006), HKT1 (Haro et al., 2005). Previous studies showed that the Na^+/H^+ anti-porter (Apse et al., 1999; Gaxiola et al., 2001), H^+-adenosine triphosphatase (H^+-ATPase) and H^+-inorganic pyro-phosphatase (H^+-PPase) (Maeshima, 2001) coordinately regulate sodium ion concentration on vacuole.

AtNHX1 gene was first identified from Arabidopsis genome, encoding Na^+/H^+ antiporter on tonoplast (Gaxiola et al., 1999). *NHX1* have been cloned from several plant species and its overexpression showed enhanced tolerance to salinity stress in transgenic plants. H^+-ATPase (V-ATPase) and H^+-inor-ganic pyrophosphatase (V-PPase) are two major electrogenic proton pump existed in plant vacuolar membrane. Both pumps create an H^+ electrochemical gradient that energize the activity of secondary active transporters, including tonoplast Na^+/H^+ antiporters. Vacuolar H^+-pyrophosphatase (V-H^+-PPase) is an enzyme that acidifies vacuole in plant cells, which maintaining vacuolar pH and catalyzing the hydrolysis of inorganic pyrophosphate (PPi) to energize proton transport from the cytoplasm into vacuoles (Maeshima, 2000). Comparison of V-H^+-PPase from different organisms has identified three conserved segment (CS). The first conserved segment (CS1) exposed to the cytosol includes the catalytic domain DVGADLVGKVE. The conserved segment (CS2) is also located in a hydrophilic loop. The third conserved segment (CS3) is in the carboxyl-terminal part containing a dozen residues. Research indicated that CS3 may be exposed to the cytosol and play a critical role in the catalytic function together with CS1 and CS2 (Rea et al., 1992; Mimura et al., 2004).

Transgenic plants overexpressing *Arabidopsis* V-H^+-PPase gene, *AVP1*, are much more tolerant to high concentrations of NaCl and to water deprivation than the non-transgenic plants (Gaxiola et al., 2001). Overexpression of *AVP1* in a commercial cultivar of tomato enhanced root system development which helps confer water deficit stress resistance in transgenic plants (Park et al., 2005). Multi-year field-trial data indicate that *AVP1*-expressing cotton leads to at least 20% more fiber yield than wild-type control plants in dry-land conditions, because of a large shoot mass in dryland conditions (Pasapula et al., 2011).

In addition to maintaining vacuo-lar pH, *AVP1* overexpression appears to facilitate auxin transport and lead to larger root system (Li et al., 2005). Recent research provided a surprising role for V-H^+-PPase gene which showed that the major function of *AVP1* in early seedling development is removal of inhibitory PPi rather than proton pump and vacuole acidi-fication (Bertoni et al., 2011; Ferjani et al., 2011). Other groups have also demonstrated that over-expression of similar genes encoding V-H^+-PPase can increase both salt- and drought tolerance in heterologous systems, including rice (Zhao et al., 2006), tobacco (Gao et al., 2006), and maize (Li et al., 2008).

In this study, we report the isolation, sequence analysis and functional characterization of *OpVP*, the V-H^+-PPase gene from *O. pumila*, and demonstrate that overexpression of *OpVP* can obviously increase salt tolerance of transgenic tobacco.

MATERIALS AND METHODS

Plant material and treatments

Seeds of *O. pumila* were collected from natural semi-arid and semi-saliferous soil located in the north of Xinjiang of China. The seeds were surface-sterilized for 20 min with 10% sodium hypochlorite, rinsed several times with sterile water and plated on square petri dishes with 0.5 × Murashige and Skoog (MS) medium (pH = 5.7) supplemented with 1% (w/v) sucrose and 0.1% (w/v) agar at 4°C for 3 days in the dark to synchronize germination. The plates were incubated in a growth chamber under a phototperiod of a 14-h/10-h light/dark cycle at 22°C. After 7 days, the seedlings were transplanted into soil and kept in a growth room with a 14-h photoperiod. When plants were 4 weeks old, the soil were watered with 0.5 × MS nutrient solution containing either 100 µM abcisic acid (ABA), or 30% PEG8000 (w/v, drought stress treatment), respectively. The leaves of these plants were collected at 0, 6, 12, 24 and 48 h after initiation of stress treatment, respectively. For the expression studies under different concentrations of NaCl treatment, 4-week-old seedlings were harvested from *O. pumila* plants after three days treatment with 0, 50, 100, 150, 200, 250, 300 and 500 mM NaCl, respectively. In the second set of experiment, 500 mM NaCl was given for different time period (0, 6, 12, 24, 48 and 72 h). All the samples were frozen immediately in liquid nitrogen and stored at -80°C until use.

Isolation of *OpVP* from *O. pumila* by RT-PCR and 5′ and 3′ RACE

To isolate the V-H$^+$-PPase gene form *O. pumila*, degenerate primers were designed based on the conserved sequenced of all known dicot V-H$^+$-PPase genes. The forward primer was 5′-SWCCWGARTGTTRTSYSTAA-3′ and reverse primer is 5′-AWGTRRCGTASTGGCWRR-3′, where R represents A or G, Y represents C or T, S represents G or C, and W represents A or T. Total RNA was isolated from the leaves of *O. pumila* plants treated with 200 mM NaCl for 8 h using the plant RNA Mini-Prep Kit (Qiagen, Hilden, Germany). Total RNA (1 µg) was used for first-strand synthesis using the M-MLV reverse transcriptase according to manufacture protocol (Promega, USA). The reverse transcription products were amplified by polymerase chain reaction (PCR) under the following thermal cycle conditions: 94°C for 2 min, followed by 35 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 90 s.

The PCR products were cloned into the pGEM T-easy vector (Promega, USA) and sequenced. Specific primers were then designed for rapid amplification of cDNA ends (RACE) according to the sequence information of the partial cDNA fragment to obtain the full-length sequence of the gene. For 5′ RACE, gene specific primers were GSP1 (5′-CAGAGGTAACAGCACCAAGAACG-3′), and GSP2 (5’-GTGCTGAATCCCTCAACAGAGCC-3′). Primers for 3′-RACE were: GSP1, 5′-TTCGCAGGCAGTTCAACACCATC-3′; and GSP2, 5′-GATTCCTCCTGGTTGCCTTGTCA-3′. The RACE reactions were performed using SMART^{TM} RACE cDNA Amplificatin Kit (Clontech, USA) according to the manufacturer’s protocol. The RACE products were cloned and sequenced. A full-length cDNA was obtained by splicing 5′-RACE fragment, partial cDNA fragment and 3′-RACE fragment into together. The full-length cDNA was used to search putative open reading frame (ORF) with ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The longest open reading frame was amplified by RT-PCR using the primers OpVPF (5′-ATGGTGGCGACAGCTTTACTACCGG-3′) and OpVPR (5′-TTAGAGGTACTTGAAAAGGATACC-3′) under the following thermal cycle conditions: 94°C for 2 min, followed by 35 cycles of 94°C for 45 s, 58°C for 45 s, and 72°C for 2 min. The resulting PCR products were cloned into the pGEM T-easy vector and positive clone was sequenced. Multiple alignments were generated using Clustal W. Phylogenetic analysis were conducted using MEGA version 4 (Tamura et al., 2007), based on the Neighbor-Joining method. Robustness of the conducted phylogentic tree was tested using 1000 bootstrap repetitions.

Expression of *OpVP*

Total RNA (1 µg) was reverse-transcribed in a sterile RNase-free microcentrifuge tube in a total volume of 10 µL with 0.5 µg oligo (dT) 18 primer, 1.0 µL dNTPs (10 mM), 2 µL 5 × M-MLV buffer, 0.25 µL RNase inhibitor (40 U/µL), and 0.5 µL reverse-transcriptase M-MLV (200 U/µL). Semi-quantitative RT-PCR was used to analyze the relative expression levels of *OpVP*. The gene-specific primers were OpVP-RTF (5′-GCCTGGGACAACGCCAAGAAGTA-3′, forward) and OpVP-RTR (5′-CACCGTGAGTGGCAAAGAAGGGA-3′, reverse). Actin-F (5′-GGTAACATTGTGCTCAGTGGTGG-3′) and Actin-R (5′-AACGACCTTAATCTTCATGCTGC-3′) were used to amplify the *Actin2* gene as an internal control. Amplification was performed for 28 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. PCR products were subsequently separated on a 1.2 % (w/v) agarose gel, then stained with ethidium bromide and photographed under UV light.

For quantitative real-time polymerase chain reaction (qRT-PCR), gene-specific primers and *Actin2* gene primers were the same as above. qRT-PCR was performed as follows: 10 ng cDNA, 5 pM of each primer and SYBR® *Premix Ex Taq* ^{TM} (Perfect Real Time) mix (TaKaRa, China) were mixed and amplified in light cycler® 480 real-time PCR system (Roche, Swtzerland) in a 20 µL final reaction volume. The threshold cycles (Ct value) of the target gene and control in different samples were obtained after qRT-PCR. Relative difference (N) was the number of treated target gene transcript copies relative to the untreated gene transcript copies, and was calculated as follows: $N = 2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct\ treated\ -\ \Delta Ct\ control)}$, where $\Delta\Delta Ct = \Delta Ct$ of the treated sample minus ΔCt of the untreated control sample, and ΔCt is the difference in threshold cycles for the *OpVP* target and the *Actin2* internal reference. For statistical analyses of gene expression among different treatments, one-way ANOVA was performed to assess significant difference between control and each treatment.

Construction of plant expression vectors and tobacco transformation

The complete ORF was amplified with the primers OpVP-P3 (5’-CGGGATCCATGGTGGCGACAGCTTTACTACCGG-3’, underline indicate the site of restriction enzyme *Kpn* Ⅰ) and OpVP-P4 (5’-GCTCTAGATTAGAGGTACTTGAAAAGGATACC-3’, underline indicate the site of restriction enzyme *Xba* Ⅰ) under the following cycle parameters: 94°C for 2 min, followed by 35 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 2 min. The resulting PCR products were cloned into the pGEM T-easy vector and positive clone was sequenced. The confirmed plasmid of pGEM T-OpVP were cut by *Kpn* Ⅰ and *Xba* Ⅰ restriction enzymes, then was inserted into the binary vector pCAMBIA2300-35S-OCS in which transgene expression was under the control cauliflower mosaic virus 35S promoter containing the neomycin phosphotransferase (*npt*Ⅱ) gene conferring resistance to kanamycin as a selectable marker. The resulting plasmid was mobilized into *Agrobacterium tumefaciens* GV3101 and transformed in tobacco (*Nicotiana tabacum* L.) with standard protocol (Horsch et al., 1986). We generated several independent homozygous transgenic tobaccos of T3 generation to be used in the following assays.

Chlorophyll content

Chlorophyll content of T3 transgenic plants grown under salt stress was estimated according to the method described by Arnon (1949). 0.1 g plant tissue was homogenized in 80% acetone and incubated in dark for 6 h. The homogenate was centrifuged at 10 000 rpm for 10 min. Supernatant obtained was read at 649 and 665 nm in Spectra Max plus-384 (Molecular device, USA) and total chlorophyll amount was calculated.

Measurement of Na^+ content

The WT and T3 transgenic tobacco plants were grown and salt stressed as described above. Leaves tissue were harvested after 7 days and 16 days of salt treatment, respectively. The leaves were dried in an oven at 60°C and their dry weights were measured till the constant weight was achieved. The dried leaves were extracted with 1 M H_2SO_4 as described by Storey (1995). The supernatants were determined using an atomic absorption spectrophotometer (Shanghai Precision & Scientific Instrument Co., China) to quantify Na^+ content.

RESULTS

Isolation and characterization of V-H$^+$-PPase (*OpVP*) gene from *O. pumila*

The full-length cDNA of *OpVP* was obtained by RT-PCR

and RACE. The *OpVP* cDNA consists of 2 698 bp, which includes 130 bp 5′ leader sequence, and 208 bp 3′-noncoding region. The open reading frame (ORF) of *OpVP* is 2 313 bp encoding a protein of 770 amino acids (GenBank accession number: KF557584) with a theroretical molecular mass of 80.7 kDa. Phylogenetic analysis (Figure 1) showed that OpVP formed a clade with the most closely related plant H^+-PPase homologs. The highest identity was found to be 98.3% similarity with *Arabidopsis lyrata*. OpVP also showed high similarity to V-H^+-PPase from *A. thaliana* AVP1 (98.2%), AVP3 (98.2%), *Brassica rapa* BrVP1 (95.2%), and *Thellungiella salsuginea* TsVP (95.2%).

Expression analysis of *OpVP* in *O. pumila*

For studying the expression analysis of *OpVP*, qRT-PCR was carried out using cDNA from different NaCl concentrations (0, 50, 100, 200, 250, 300 and 500 mM) treated plants (Figure 2A). In the absence of NaCl condition, expression of *OpVP* was low. When treated with different concentrations of NaCl for three days, *OpVP* transcripts increased significantly as the NaCl concentration raised ($P < 0.05$), indicating that the expression of *OpVP* was induced by salt stress (Figure 2A). The expression of *OpVP* in leaves of *O. pumila* plants corresponding to the different time period was also measured at 500 mM NaCl treated (Figure 2B). The results showed that the transcript did not increased at 6 h, but gradually increased from 12-72 h, and reached at a maximum at 48 h ($P < 0.05$).

The expression profile of *OpVP* in seedlings of *O. pumila* was investigated in the presence of 20% PEG8000, 1 µM abcisic acid (ABA), 1 µM indo-3-aceticacid (IAA) and 2 µM gibberellins (GA3) by semiquantitative RT-PCR. The result demonstrated that drought stress, ABA application, IAA and GA3 induced transcription accumulation of *OpVP* (Figure 3). In the drought stress treatment, *OpVP* transcripts were detected at 0 h and induced at 1 h, and reached a peak at 2 h of treatment, after that gradually decreased (Figure 3A). Interestingly, the transcript level of *OpVP* also increased in response to exogenous ABA, which functions as a signaling molecule and plays an important role in stress tolerance.

Figure 3B showed that the transcript level of *OpVP* under 1 µM ABA induction was up-regulated, and reached a maximum at 2 h of 1 µM ABA treatment, and decreased only slightly after 48 h of treatment. In addition, the expression profiles of *OpVP* response to different concentration of IAA and GA3 were also investtigated. To our surprise, both IAA and GA3 can intensively induce the expression of *OpVP*. *OpVP* transcripts arrived at high value at 2 h of 1 µM IAA or 2 µM GA3 of treatment, and maintained high expression level until 12 h (Figure 3C and D).

Overexpression of *OpVP* confers enhanced salt tolerance in transgenic tobacco

To investigate the function of *OpVP*, after transformation of tobacco plants with overexpression vector *p35S:OpVP* and selection with the kanamycin, several individual T3 transformants were produced. The expression of *OpVP* gene in all transgenic lines has been monitored by semi-quantitative RT-PCR. Figure 4A showed that *OpVP* were all expressed in 5 independent transgenic lines, however, L4 displayed much higher level of expression of *OpVP* than the four other lines. L2 and L4 were used for subsequent analysis. To assess the effect of salt tolerance, the seedling of both WT and transgenic plants were planted on soil pots. 4-week plants were subjected to incubation of 200 mM NaCl. Seven days later, all the plants showed no obvious effect, but some base rosette of WT turned white and died whereas the leaves in transgenic plants could continue to expand leaves and greening (figures were not supplied). After treated consecutively by 200 mM NaCl for 45 days, the WT plants grown weak and flowering, and the leaves became chlorotic, whereas the transgenic plants still remained in vegetative period and the leaves were green (Figure 4B).

To further evaluate the increased salt tolerance of transgenic tobacco overexpressing *OpVP*, chlorophyll contents of wild-type and both transgenic lines was determined. Chlorophyll contents were measured both in WT and transgenic plants of L2 and L4 stressed by 200 mM NaCl for 0, 7, 16 and 45 day, respectively. The wild type and the transgenic line L4 showed the same chlorophyll content, however, the transgenic line L2 showed higher chlorophyll content before salt stress ($P < 0.05$) (Figure 5). The contents of chlorophyll in all the experiment lines increased on 7 days. There were more chlorophyll contents in wild-type than both transgenic lines ($P < 0.05$). When stressed from 7 to 45 days, the chlorophyll content in WT and L2 plants reduced significantly with the stress time increase. However, the chlorophyll content in L4 plants reduced gradually, but it increased significantly when stressed to 45 days ($P < 0.05$). For example, after seven days of salt stress, there was 77% increase of the chlorophyll in wild-type, whereas only 40 and 61% in transgenic L2 and L4, respectively. However, after 45 days of salt stress, there was 50% increase of chlorophyll in L4, whereas there was 66% reduction in wild-type, and 50% reduction in L2. Comparatively, the chlorophyll content in L4 plants were highest at 45 days of salt stress, which is also correlated with the expression levels of *OpVP*.

Na^+ accumulation in transgenic plants

The contents of Na^+ were determined in the leaves of plants from transgenic lines and wild-type grown under 200 mM NaCl treatments (0 and 16 days). In the absence

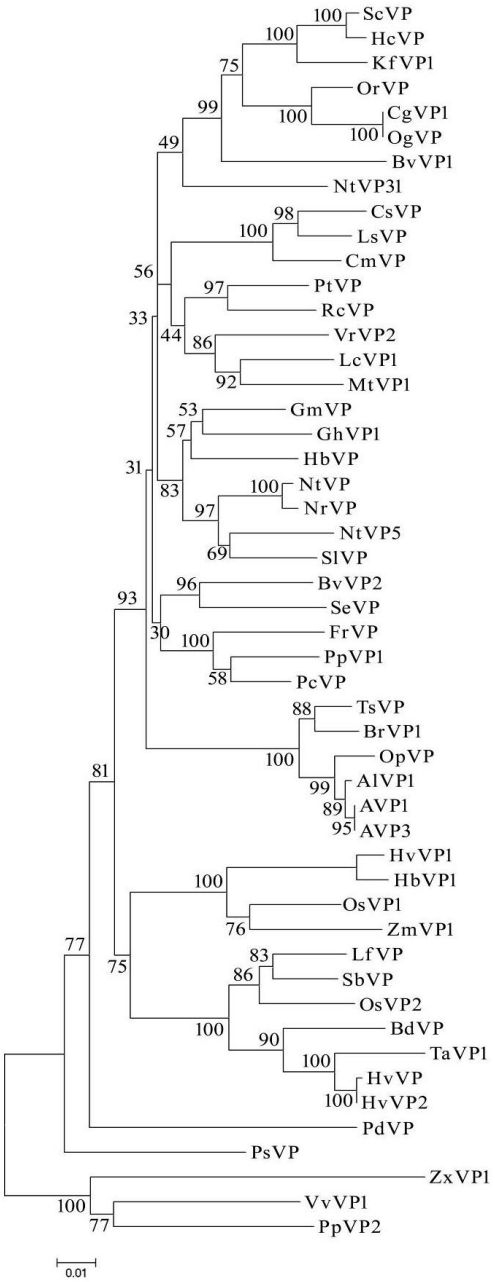

Figure 1. Phylogentic analysis of OpVP with other known plant H^+-pyrophosphatase homologs. Phylogentic analysis was based on typical vacuolar proton-pumping PPase from plants. The gene abbreviation and GenBank accession number are as the follows: *Suaeda corniculata* (ScCP; ADQ00196), *Halostachys caspica* (HcVP; ABO45933), *Kalidium foliatum* (KfVP1, ABK91685), *Oxybasis rubra* (OrVP; AAM97920), *Chenopodium glaucum* (CgVP1; ABD98735), *Oxybasis glauca* (OgVP; ABD98735), *Beta vulgaris* (BvVP1; AAA61610), *Beta vulgaris* (BvVP2; AAA61609), *Nicotiana tabacum* (NtVP; CAA58701), *Nicotiana tabacum* (NtVP5; CAA54869), *Nicotiana tabacum* (NtVP31; CAA58700), *Cucumis sativus* (CsVP; XP_004150506), *Lagenaria siceraria* (LsVP; AET95912), *Cucurbita moschata* (CmVP; BAA33149), *Populus trichocarpa* (PtVP; XP_002325187), *Ricinus communis* (RcVP; XP_002530755), *Vigna radiata* (VrVP2; BAA23649), *Lotus corniculatus* (LcVP1; ABS01290), *Medicago truncatula* (MtVP1; ACI22377), *Glycine max* (GmVP; XP_003531725), *Gossypiym hirsutum* (GhVP1; ADN96173), *Hevea brasiliensis* (HbVP; AAS66771), *Nicotiana rustica* (NrVP; ABF85694), *Solanum lycopersicum* (SlVP; XP_004241690), *Salicornia europaea* (SeVP; AEI17666), *Fragaria vesca* (FrVP; XP_004303283), *Prunus persica* (PpVP1; AAL11506), *Prunus persica* (PpVP2; AF367447), *Pyrus communis* (PcVP; BAC41250), *Thellungiella salsuginea* (TsVP; AAR08913), *Brassica rapa* (BrVP1; AET95910), *Olimarabidopsis pumila* (OpVP; KF557584), *Arabidopsis lyrata* (AlVP1; XP_002890120), *Arabidopsis thaliana* (AVP1; NP_173021), *Arabidopsis thaliana* (AVP3; AAA32754), *Hordeum vulgare* (HvVP; ACA63883), *Hordeum vulgare* (HvVP1; BAB18681), *Hordeum vulgare* (HvVP2; BAA02717), *Hordeum brevisubulatum* (HbVP1; AAP06752.1), *Oryza sativa* (OsVP1; BAA08232), *Oryza sativa* (OsVP2; BAA08233), *Zea mays* (ZmVP1; NP_001105380), *Leptochloa fusca* (LfVP; ACT98610), *Sorghum bicolor* (SbVP; ADJ67258), *Brachypodium distachyon* (BdVP; 003564217), *Triticum aestivum* (TaVP1; AAP55210), *Potamogeton distinctus* (PdVP; BAF63470), *Picea sitchensis* (PsVP, ABR18024), *Zygophyllum xanthoxylum* (ZxVP1; ABU92563), *Vitis vinifera* (VvP1; AAF69010).

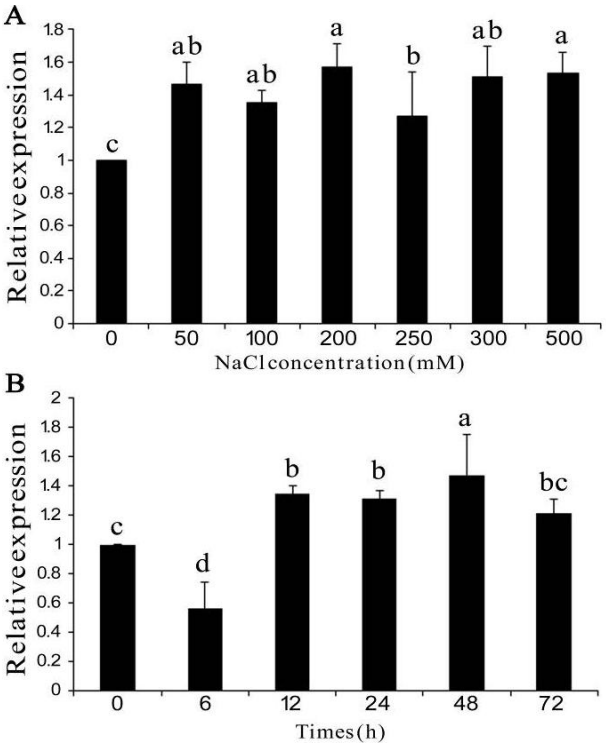

Figure 2. qRT-PCR analysis of the *OpVP* gene under NaCl stress condition. **A,** Different concentration of NaCl was given for 72 h. **B,** 500 mM NaCl for different time periods (h). Values are means ± SE. Similar letters indicate no significant difference at $P < 0.05$ using Duncan test.

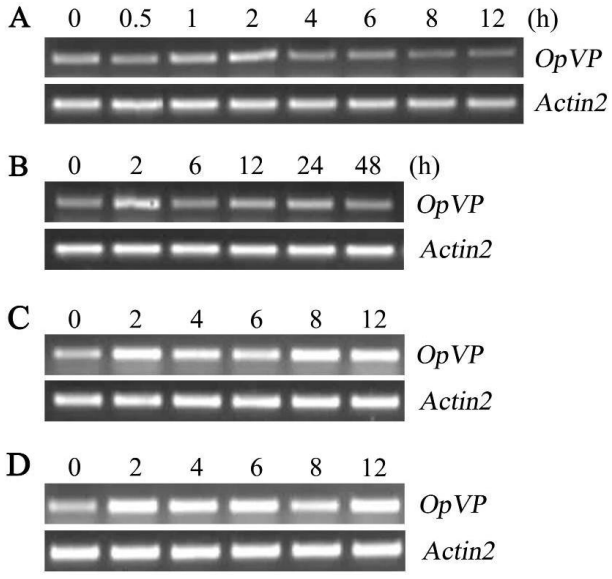

Figure 3. Expressing profiles of *OpVP* in *O. pumila* seedlings in reponse to different stress and hormone induction. **A,** Expressing pattern of *OpVP* in the whole plant under dehydration 20% PEG8000. **B,** Expressing pattern of *OpVP* under 1 µM ABA. **C,** Expressing pattern of *OpVP* under 1 µM IAA; D, Expressing pattern of *OpVP* under 2 µM GA3.

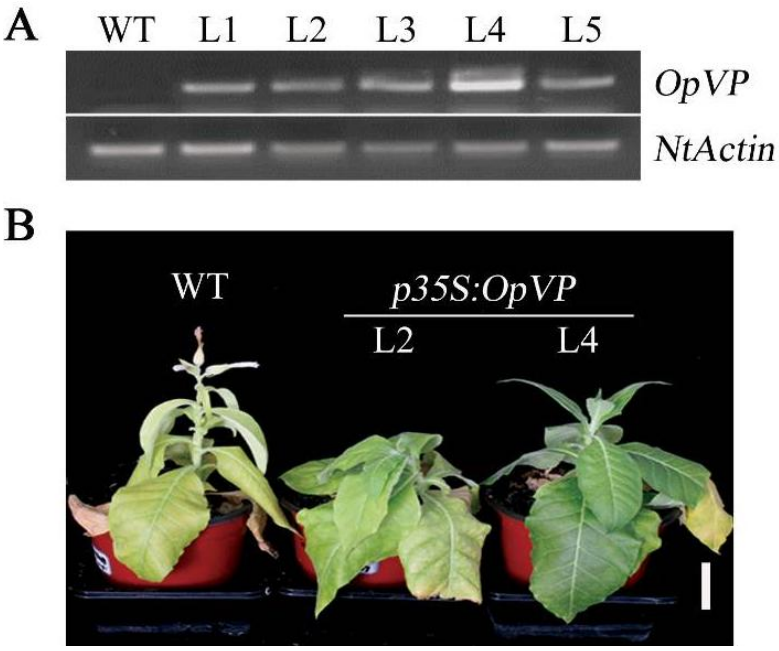

Figure 4. Analysis of *OpVP* transgenics (T3). **A,** Analysis of *OpVP* expression in tobacco transgenic lines by semiquantitative RT-PCR. **B,** Growth comparison of 45-day-old seedlings stressed by 200 mM NaCl. The photograph shows plants at the 45th day after treatment with 200 mM NaCl. WT: non-transgenic controls; L2, L4: transgenic plants overexpressing *OpVP*.

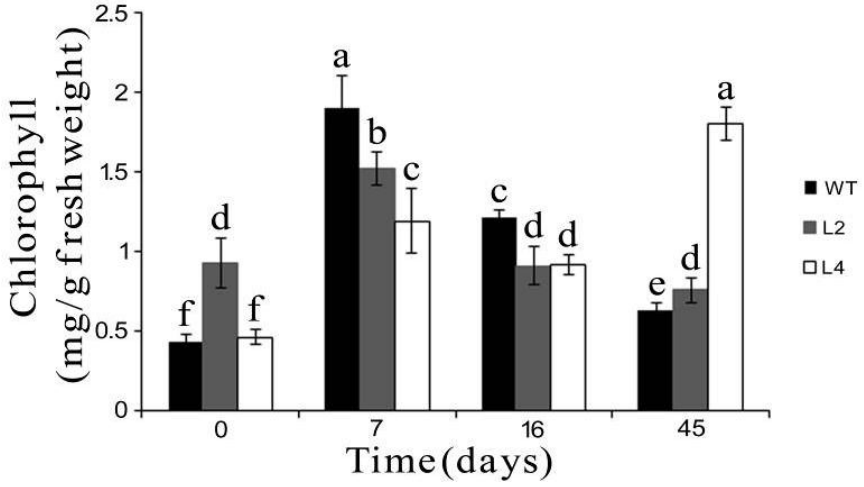

Figure 5. Chlorophyll content in the leaf of transgenic and wild type (WT) under 200 mM NaCl stress.The chlorophyll contents were measured 0, 7, 16 and 45 days salt treatments. Values represents means ± S.E. Columns with different letters indicate significance at $P < 0.05$ (Duncan test). WT, Non-transgenic controls; L2, L4: transgenic plants overexpressing *OpVP*.

of NaCl, there were no significant differences in Na^+ concentration between WT and transgenic plants. When stressed at 200 mM NaCl, Na^+ content significantly increased in the leaves of both transgenic and WT plants (Figure 6). When stressed to 16 days, however, there was significantly more Na^+ in both L2 and L4 than wild-

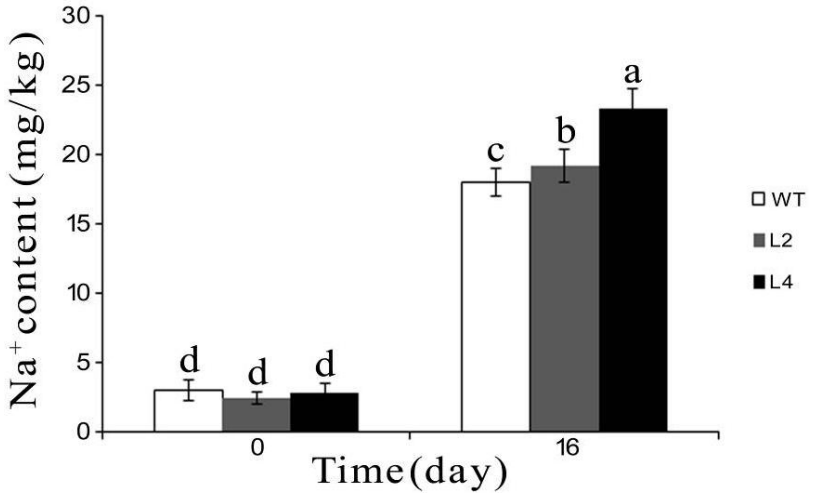

Figure 6. Na^+ contents in the leaf of transgenic and wild type (WT) under 200 mM NaCl stress. The Na^+ contents were measured 0 and 16 days of salt treatments. Values are means ± S.E. Columns with different letters indicate significance at $P < 0.05$ (Duncan test). WT, Non-transgenic controls; L2, L4: transgenic plants overexpressing *OpVP*.

type ($P < 0.05$). Comparatively, the contents of Na^+ in L4 plants were highest under salt stress, which is also correlated with the expression levels of *OpVP*. These results suggest that the enhanced salt tolerance in transgenic plants might be related to an increased Na^+ accumulation capacity.

DISCUSSION

V-H^+-PPase plays an important role in the maintenance of the pH gradient across the vacuole membrane in plant stress response. In this study, a novel gene encoding a V-H^+-PPase, *OpVP*, was isolated from ephemeral plant *O. pumila*. An amino acid comparison between other Brassicaceae revealed that the polypeptides are well conserved. This indicates the V-H^+-PPase is one of the most highly conserved polypeptides among higher plants.

Expression of *OpVP* in *O. pumila* seedlings was significantly induced by salinity, drought and ABA application (Figures 2 and 3). This pattern of expression is similar to that of *TsVP* in *T. halophila* (Lv et al., 2008), *ScVP* in *S. corniculata* (Liu et al., 2011), *HVP1* and *HVP10* in barley (Fukuda et al., 2004). In addition, the expression level of *OpVP* was also significantly induced by IAA and GA3 (Figure 3), suggesting that gene may function differently under hormone treatment. Further studies are now in progress in our laboratory to investigate this relationship between hormone and *OpVP*.

To avoid the toxic effect of salt, plants have developed mechanisms to limit Na^+ uptake, to increase Na^+ exclusion, or to sequester Na^+ into vacuoles (Amtmann and Sanders, 1999). The vacuolar H^+-pumps play a key role in the maintenance of the H^+ electrochemical gradient across the vacuolar membrane. H^+-PPase can enhance the accumulation of Na^+ in vacuoles. Accumulation of Na^+ in vacuoles instead of in the cytoplasm can avoid the toxic effect of excessive Na^+ in plant cells (Gaxiola et al., 2001). V-H^+-PPase is a primary electrogenic proton pump that translocates protons across the tonoplast into vacuoles, thereby supporting strong ion differences and membrane polarization (Felle 2005), and alleviating the acidification of the cytosol (Stitt, 1998; Maeshima, 2000). Proton pumps coordinating with Na^+/H^+ antiporters on tonoplast enhance the ability of Na^+ uptake, as a result of accumulating more Na^+ on vacuole (Apse et al., 1999). Overexpression of *OpVP* gene in tobacco leads to increasing the activity of a H^+ pump on the vacuolar membrane of tobacco to move more H^+ into the vacuoles, therefore generating a higher proton electrochemical gradient ($\Delta\mu H^+$) that can be used to energize Na^+/H^+ antiporters. Enhanced expression of the vacuolar proton pumps should increase vacuolar solute accumulation by increasing the availability of protons. The sequestration of ions such as sodium in the vacuole could confer salt tolerance. In the present work, overexpressing *OpVP* transgenic tobacco showed a tendency to accumulate more Na^+ under salt stress conditions than non-transgenic wild type (Figure 6). The sequestration of Na^+ in the vacuole instead of in cytoplasm may prevent Na^+ toxicity, thus increases salt tolerance of transgenic tobacco plants (Figure 4). Moreover, *OpVP*-expressing tobacco plants maintained more chlorophyll content than wild-type control plants did under salt stress condition (Figure 5).

In conclusion, we successfully isolated and characte-

rized the *OpVP* cDNA from emphemeral plants *O. pumila* and developed transgenic *OpVP* expressing tobacco plants. The results indicated that overexpression of *OpVP* gene can increase salt tolerance by increasing the capacity to accumulate Na^+ in the vacuole. The overexpression of *OpVP* gene in economically important crop plants might be a strategy for engineering cultivars of agriculturally important plants to improve salinity tolerance in crops in salinization areas of the world.

ACKNOWLEDGEMENTS

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African Journal of Biotechnology

Full Length Research Paper

In vitro regeneration of selected Kenyan papaya (*Carica papaya* L.) lines through shoot tip culture

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Papaya, an important fruit crop in Kenya is commercially propagated through seeds which lead to production of non-true-to-types plants. Conventional vegetative propagation methods are not carried out, hence, the need for micropropagation for mass multiplication of selected lines. An assessment for the effect of 6-benzylaminopurine (BAP) at 0.1, 0.5, 1.0 and 2.0 mg/l combined with α-naphthalene acetic acid (NAA) at 0.05, 0.1, 0.5 and 1.0 mg/l and a control on shoot multiplication and elongations, and indole-3-butyric acid (IBA) at 0, 0.1, 0.5, 1.0, 2.0, 2.5 and 3.0 mg/l on root induction were evaluated. Number of shoots and their length were recorded every three weeks for 12 weeks. Number of roots, root length and percentage rooting induction were recorded after eight weeks. The highest number of shoots was recorded in 0.5 mg/l BAP combined with 0.1 mg/l NAA and the longest shoots were recorded in 0.1 mg/l BAP combined with 0.05 mg/l NAA across the three lines. IBA at 2.5 mg/l produced the highest number of roots, root length and highest percentage of rooting induction. An *in vitro* regeneration of selected papaya lines through shoot tip culture was established.

Key words: *Carica papaya*, *in vitro* regeneration, shoots multiplication, rooting.

INTRODUCTION

Papaya (*Carica papaya* L.) is a fruit commonly eaten fresh all over the world. The ripe fruit is low in calories and rich in vitamins A and vitamin C (Farzana et al., 2008). Papain, a proteolytic enzyme present in the latex of green fruits, has many uses in beverages, food and pharmaceutical industries. These uses include chill-proofing beer, tenderizing meat and in drug preparation for alleviating digestive ailments (Nakasone and Paull, 1998). In Kenya, papaya is a widespread fruit crop especially where enough water is available for its cultivation (Imungi and Wabule, 1990). The fruit crop is believed to have been introduced into Kenya more than 50 years ago. The main varieties grown are hawaii, solo, honeydew, sunrise, and local types. These varieties were introduced from Hawaii, Philippines, India and Indonesia (Kamau et al., 1993).

Sexual propagation is the commercial method of propagation for papaya. Being heterozygous and a cross pollinated crop, sexual propagation has resulted in immense variation among populations for yield, size, shape, quality of fruit and disease susceptibility leading to production of non-true-to-type plants (Panjaitan et al., 2007). The signi-

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Abbreviations: BAP, 6-benzylaminopurine; **NAA,** naphthalene acetic acid; **IBA,** indole-3-butyric acid.

ficance of vegetative propagation in the maintenance of genetic uniformity and preservation of identity of an elite clone or cultivar is well recognized in horticultural crops. To develop true-to-type plants, an efficient vegetative propagation technique is required.

Conventional vegetative propagation methods in papaya such as grafting (Allan et al*.,* 2010) and rooted cuttings (Rajan and Markose, 2007) exist, but they are often not carried out on a large scale. Hence, the alternative method is micropropagation techniques for mass multiplication of elite materials. Development of an efficient *in vitro* regeneration system would be a remarkable progress for mass propagation and uniform plants for both commercial and research purposes.

Papaya is most commonly propagated *in vitro* by shoot tip or axillary bud (culture (Teixeira da Silva et al*.,* 2007). As such several protocols for *in vitro* plantlet regeneration from shoot tips of papaya have been developed in other regions of the world (Kabir et al., 2007; Panjaitan et al., 2007).

However, most of the protocols are genotype dependent and therefore cannot be reproduced (Mishra et al*.,* 2007). There are no documented studies on micropro-pagation of papaya lines in Kenya. The objective of this study was to develop an efficient *in vitro* regeneration system for micropropagation of Kenyan papaya lines through shoot tip culture.

MATERIALS AND METHODS

Plant material and sterilization procedure

Three local papaya lines namely line 1; line 2 and line 3 were selected from an existing papaya breeding project based at Jomo Kenyatta University of Agriculture and Technology (JKUAT) in Juja. The selection criteria were based on plant height, fruit yield and sex of mother plant. Seeds were extracted and established in greenhouse as stock plants. 1 cm shoot tips (explants) were harvested from three month seedlings and placed in a glass beaker and kept in running tap water for 30 min to remove physical impurities. Under a clean lamina flow hood, the explants were subjected to 70% (v/v) ethanol for 30 s and rinsed with double distilled water thrice to remove ethanol and then subjected to 20% (v/v) household bleach (jik®) containing 3.85% sodium hypochlorite and 100 µl/l Tween 20® for 20 min then rinsed three times with double distilled water.

Shoot multiplication and elongation

After sterilization, the peripheral surfaces of the tissues were trimmed leaving about 0.7 cm long explant which were cultured on Murashige and Skoog (MS) (1962) basal medium, supplemented with 30 g/l sucrose, and 6-benzylaminopurine (BAP) at concentrations 0.1, 0.5, 1.0 and 2.0 mg/l combined with naphthalene acetic acid (NAA) at concentrations 0.05, 0.1, 0.5 and 1.0 mg/l and a control where no plant growth regulator was applied for shoot multiplication and elongation, solidified with 2.5 g/l gerlite. Each concentration was replicated four times. The number of shoots produced per explant and their length were recorded after every 3 weeks for a period of 12 weeks. The explants were subcultured in a fresh media after every three weeks.

Root induction

Proliferated shoots, about 3 cm long were separated into individual explant and method described by Tsong et al. (2000) with modifications was used. The shoots were first subcultured in full strength MS containing 3% (w/v) sucrose, 0.28% gerlite supplemented with indole-3-butyric acid (IBA) at concentrations 0, 0.1, 0.5, 1.0, 2.0, 2.5, 3.0mg/l IBA for one week in darkness for root induction and thereafter transferred into vermiculite supplemented with half strength MS supplemented with 3% (w/v) sucrose for further root development. The experiment was replicated three times. Number of roots per explants, their root length and the rate (%) of shoots that produced roots were determined after 8 weeks.

Experimental design and statistical analysis

The experiments were carried out in factorial design with two factors (three papaya lines and varied hormonal treatments). Data collected was analyzed using analysis of variance (ANOVA) and means differing significantly were compared using student Newman Keuls (SNK) test at $p \leq 0.05$.

Acclimatization

Rooted plants were taken out from the culture bottles and washed carefully under running tap water for complete removal of remains of the medium. Pots (9x6 cm) were kept ready filled with forest soil, manure, sand and vermiculite in the proportion of 2:1:1:1, respectively. All the pots were covered with two layers of shade net, which was kept moist for providing high humidity to the plants. After one week, one layer of shade net was removed while the other layer was removed after another one week. Within three weeks in acclimatization chamber, *in vitro* regenerated plantlets had hardened and were taken to greenhouse for further growth.

RESULTS

Within seven days after culture initiation, new leaves started forming from the shoot tip (Figure 1A). After 28 days of culture, a number of axillary and adventitious buds emerged out of shoot tip (Figure 1B). Later, many of these buds developed as young shoots. The multiple shoots developed further after 12 weeks of culture (Figure 1C). In this study, different concentrations of BAP combined with NAA tested affected the mean number of shoot produced and mean shoot length per shoot tip. Increase in BAP up to 0.5 mg/l and NAA up to 0.1 mg/l increased the mean number of shoots produced per shoot tip (Table 1). However, the mean number of shoots produced per explant decreased on increasing the BAP concentration up to 1.0 mg/l and NAA up to 0.5 mg/l across all lines (Table 1). Explants cultured in absence of BAP and NAA, most of them senesced without producing shoots while other produced an average of 1 shoots per explants across the three lines. The mean number of shoots produced per shoot tip was influenced by interaction between papaya lines and BAP and NAA concentrations used ($P<0.05$). In papaya line 1, MS media supplemented with 0.5 mg/l BAP combined with 0.1 mg/l NAA produced the highest mean number of

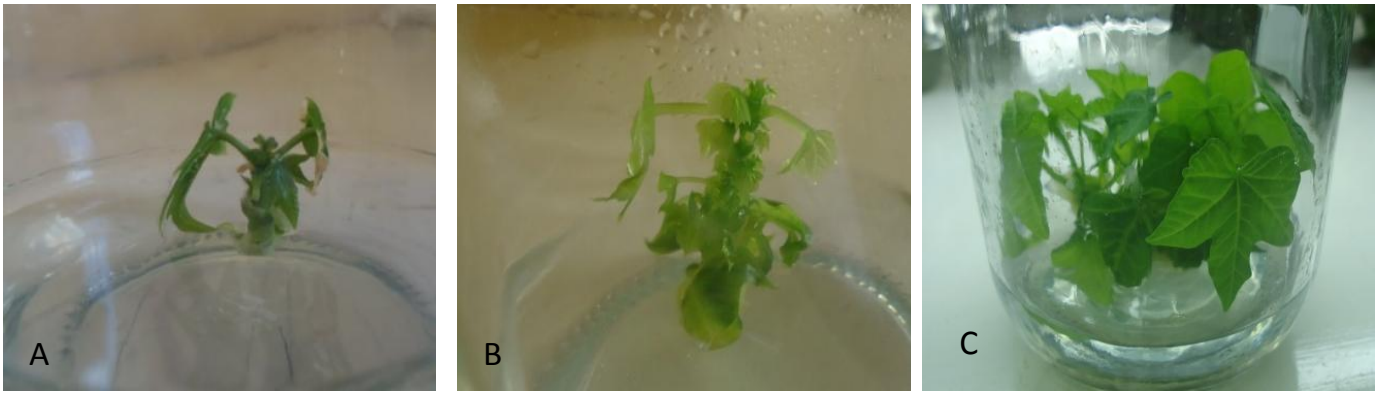

Figure 1. *In* vitro shoot tip development. A, Formation of leaves on shoot tips seven days after culture; B, shoot proliferation from shoot tips 28 days after culture; C, development of multiple shoots12 weeks of culture.

Table 1. Effect of different concentrations of BAP combined with NAA on mean number of shoot per shoot tip of three papaya lines(cm) after 12 weeks of culture, n=153.

BAP + NAA (mg/l)	Mean number of shoot		
	Line 1	Line 2	Line 3
0+0	0.8 ± 0.16^{h}	1.0 ± 0.44^{g}	0.6 ± 0.21^{h}
0.1+0.05	11.8 ± 0.91^{c}	8.8 ± 0.30^{c}	16.5 ± 1.54^{b}
0.5+0.05	13.8 ± 0.70^{b}	13.6 ± 0.95^{b}	16.8 ± 0.94^{b}
1.0+0.05	8.5 ± 0.22^{ef}	8.8 ± 0.47^{c}	6.8 ± 0.60^{efg}
2.0+0.05	6.5 ± 0.50^{fg}	7.8 ± 0.37^{cd}	6.5 ± 0.88^{efg}
0.1+0.1	11.0 ± 0.51^{cd}	12.3 ± 0.84^{b}	14.3 ± 0.91^{c}
0.5+0.1	24.3 ± 0.95^{a}	25.8 ± 2.08^{a}	19.3 ± 0.98^{a}
1.0+0.1	8.1 ± 0.47^{ef}	9.0 ± 0.25^{c}	10.6 ± 0.33^{d}
2.0+0.1	5.0 ± 0.36^{g}	7.6 ± 0.33^{cd}	5.8 ± 0.60^{efg}
0.1+0.5	4.1 ± 0.40^{g}	4.2 ± 0.30^{ef}	4.1 ± 0.40^{fg}
0.5+0.5	8.3 ± 0.66^{ef}	8.6 ± 0.21^{c}	8.5 ± 0.71^{de}
1.0+0.5	9.6 ± 0.49^{de}	8.1 ± 0.60^{cd}	8.8 ± 0.30^{de}
2.0+0.5	5.5 ± 0.42^{g}	5.8 ± 0.47^{de}	7.1 ± 0.65^{ef}
0.1+1.0	4.5 ± 0.34^{g}	2.3 ± 0.21^{fg}	4.6 ± 0.21^{fg}
0.5+1.0	4.1± 0.30^{g}	2.8 ± 0.40^{fg}	3.8 ± 0.30^{g}
1.0+1.0	4.6 ± 0.6^{g}	4.8 ± 0.54^{ef}	6.6 ± 0.49^{efg}
2.0+1.0	5.5 ± 0.42^{g}	4.0 ± 0.36^{ef}	5.6 ± 0.33^{efg}

Mean values within a column followed by the same letter are not significantly different by SNK (P≤ 0.05).

shoots of 24.5 within 12 weeks of sub culturing, followed by MS media supplemented with 0.5 mg/l BAP combined with 0.05 mg/l NAA with a mean of 13.8 shoots (Table 1). Control had the least mean number of shoots produced per explant with a mean of 0.8 shoots (Table 1).

In papaya line 2, the highest mean number of shoots produced per shoot tip was recorded in MS media supplemented with 0.5 mg/l BAP combined with 0.1mg/l NAA with a mean of 25.8 shoots per explant. This was followed by MS media supplemented with 0.5 mg/l BAP combined with 0.05 mg/l NAA with a mean of 13.6 shoots per explant and MS media supplemented with 0.1 mg/l BAP combined with 0.1 mg/l NAA with a mean of 12.3 shoots per explant. Control recorded the least with a mean of 1 shoot per explant (Table 1).

In papaya line 3, MS media supplemented with 0.5 mg/l BAP combined with 0.1 mg/l NAA recorded the highest number of shoots produced per explant with a mean 19.3 shoots. This was followed by MS media supplemented with 0.1 mg/l BAP combined with 0.05 and 0.5 mg/l BAP

Table 2. Effect of different concentrations of BAP combined with NAA on mean shoot length (cm) of three papaya lines after 12 weeks of culture, n=153.

BAP + NAA (mg/l)	Mean shoot length (cm)		
	Line 1	Line 2	Line 3
0+0	1.31 ± 0.017^{g}	1.38 ± 0.031^{g}	1.58 ± 0.033^{f}
0.1+0.05	3.25 ± 0.085^{a}	3.30 ± 0.082^{a}	3.28 ± 0.070^{a}
0.5+0.05	2.63 ± 0.042^{b}	3.03 ± 0.105^{b}	3.01 ± 0.087^{b}
1.0+0.05	1.80 ± 0.037^{de}	2.05 ± 0.043^{d}	1.73 ± 0.042^{e}
2.0+0.05	1.76 ± 0.049^{e}	1.68 ± 0.031^{ef}	1.60 ± 0.037^{ef}
0.1+0.1	2.71 ± 0.048^{b}	2.78 ± 0.079^{c}	2.16 ± 0.033^{c}
0.5+0.1	2.26 ± 0.042^{c}	2.11 ± 0.031^{d}	2.13 ± 0.056^{c}
1.0+0.1	1.70 ± 0.026^{ef}	2.10 ± 0.058^{d}	2.13 ± 0.056^{c}
2.0+0.1	1.43 ± 0.033^{g}	1.83 ± 0.033^{e}	1.96 ± 0.071^{d}
0.1+0.5	1.30 ± 0.026^{g}	1.51 ± 0.031^{fg}	1.73 ± 0.021^{e}
0.5+0.5	1.80 ± 0.026^{de}	1.80 ± 0.086^{e}	1.68 ± 0.031^{ef}
1.0+0.5	1.81 ± 0.031^{de}	1.80 ± 0.037^{e}	1.75 ± 0.022^{e}
2.0+0.5	1.85 ± 0.022^{de}	1.70 ± 0.037^{ef}	1.80 ± 0.037^{e}
0.1+1.0	1.56 ± 0.021^{f}	1.50 ± 0.026^{f}	1.58 ± 0.040^{ef}
0.5+1.0	1.71 ± 0.031^{ef}	1.63 ± 0.021^{ef}	1.68 ± 0.037^{ef}
1.0+1.0	1.95 ± 0.043^{d}	1.88 ± 0.040^{e}	1.68 ± 0.0373^{f}
2.0+1.0	1.70 ± 0.068^{ef}	1.70 ± 0.082^{ef}	1.73 ± 0.049^{e}

Mean values within a column followed by the same letter are not significantly different by SNK ($P \leq 0.05$).

combined with 0.05 mg/l NAA with a mean of 16.5 and 16.8 shoots, respectively. Control exhibited the least number of shoots with a mean of 0.6 shoots (Table 1). The mean shoot length (cm) per shoot tip was influenced by interaction between papaya lines and BAP and NAA concentrations used (P<0.05).

In papaya line 1, the longest shoot was recorded in MS media supplemented with 0.1 mg/l BAP and 0.05 mg/l NAA with a mean of 3.25 cm. This was followed by MS media supplemented with 0.1 mg/l BAP, 0.1 mg/l NAA, 0.5 mg/l BAP and 0.05 mg/l NAA with a mean of 2.71 and 2.63 cm, respectively.

The least shoot length was recorded in control with a mean of 1.31cm (Table 2). In papaya line 2, the longest shoot was recorded in MS media supplemented with 0.1 mg/l BAP and 0.05 mg/l NAA with a mean of 3.3 cm. This was followed by MS media supplemented with 0.5 mg/l BAP and 0.05 mg/l NAA with a mean of 3.0 cm. The least shoot length was recorded in control with 1.38 cm (Table 2).

In papaya line 3, the longest shoot was recorded in MS media supplemented with 0.1 mg/l BAP and 0.05 mg/l NAA with a mean of 3.28 cm. This was followed by MS media supplemented with 0.5 mg/l BAP and 0.05 mg/l NAA with a mean of 3.01 cm. The least shoot length was recorded in control with a mean of 1.5 cm (Table 2).

Root development was induced by pre-treating shoots in IBA for one week in darkness, followed by culture on vermiculite supplemented with half strength MS medium within 8 weeks (Figure 2). Meanwhile, shoots without IBA pre-treatment placed on medium with vermiculite supplement did not produce roots at all.

The rate of root induction was influenced by the interaction between papaya lines and IBA concentrations (P<0.05) used. IBA at 2.5 mg/l recorded the highest rate of root induction within 8 weeks across the three papaya lines with 75, 83 and 55% in papaya lines 1, 2 and 3, respectively (Table 3).

Significant effect of IBA concen-trations on the mean number of roots produced per shoot and the average root length (p<0.05) was recorded. IBA at 2.5 mg/l produced the highest number of roots and the longest root length within eight weeks with an average of 5.55 roots per shoot and mean root length of 2.46 cm (Table 4). After three weeks in acclimatization greenhouse, *in vitro* regenerated plantlets were ready for transplanting (Figure 3).

DISCUSSION

The production of shoots from axillary buds and shoot tip explants is the most reliable method of *in vitro* propagation of papaya (Teixeira da Silva et al., 2007). In this study, an *in vitro* regeneration system of three papaya lines through shoot tip culture was attempted. Different concentrations of BAP combined with NAA tested

Figure 2. Root formation in vermiculite supplemented with half strength MS 8 weeks after culture.

Table 3. The effects of different concentrations of IBA on the proportion of shoots (%) that rooted within 8 weeks, n=63.

Concentration of IBA (mg/l)	Proportion of shoots producing roots (%)		
	Papaya line 1	Papaya line 2	Papaya line3
0	0^{c}	0^{d}	0^{c}
0.1	5.66 ± 1.6^{c}	17.0 ± 1.5^{c}	5.667 ± 1.56^{c}
0.5	33.0 ± 4.08^{b}	33.0 ± 4.08^{bc}	33.0 ± 4.08^{b}
1.0	35.0 ± 5.2^{b}	33.0 ± 5.2^{bc}	33.0 ± 4.06^{b}
2.0	38.6 ± 5.6^{b}	38.6 ± 5.6^{b}	38.6 ± 5.6^{b}
2.5	72.3 ± 5.3^{a}	83.3 ± 9.5^{a}	55.6 ± 5.6^{a}
3.0	37.3 ± 4.88^{b}	33.0 ± 5.3^{bc}	33.0 ± 5.3^{b}

Mean values within a column followed by the same letter are not significantly different by SNK ($P \leq 0.05$).

Table 4. The effects of different concentrations of IBA on mean number of roots and the mean root length per shoot within 8 weeks of culture n=63.

Concentration of IBA (mg/l)	Mean number of roots	Mean root length (cm)
0	0 ± 0^{e}	0.00 ± 0^{e}
0.1	1.55 ± 0.557^{d}	0.50 ± 0.067^{d}
0.5	2.22 ± 0.333^{cd}	0.93 ± 0.120^{c}
1.0	3.11 ± 0.333^{c}	1.21 ± 0.058^{bc}
2.0	5.0 ± 0.577^{b}	1.16 ± 0.088^{bc}
2.5	5.5 ± 0.667^{a}	2.46 ± 0.120^{a}
3.0	4.88 ± 0.577^{b}	1.37 ± 0.115^{b}

Mean values within a column followed by the same letter are not significantly different by SNK ($P \leq 0.05$).

Figure 3. In *vitro* regenerated papaya plantlets.

affected number of shoot per shoot tip as well as shoot length. Increase in BAP up to 0.5 mg/l and NAA up to 0.1 increased the mean number of shoots produced per shoot tip. However, the mean number of shoots produced per explant decreased on increasing the BAP concentration from 0.5 to 1.0 mg/l and NAA from 0.1 to 0.5 mg/l among three lines.

The probable reason for this could be BAP at 0.5 mg/l and NAA at 0.1 mg/l were the optimum concentrations for shoot multiplication. Panjaitan et al. (2007) reported that BAP concentration more than 1.0 mg/l supported poor rate of shoot multiplication in field growth shoot tips of hermaphrodite papaya (*C. papaya* L. cv. Eksotika). Besides, excessive BAP has been shown to inhibit shoot growth and reduce proliferation rates in papaya (Drew, 1988).

There were statistically significant interaction between papaya lines and BAP and NAA concentrations used on the mean number of shoots produced per explant and mean shoot length (cm). Significant interaction between papaya lines and IBA concentration on rate of shoots (%) that produced roots was also noted. This shows papaya lines differences. Such interactions between papaya lines and hormonal treatment influences have been reported in tissue culture of papaya (Litz and Conover, 1977).

BAP at 0.5 mg/l combined with NAA at 0.1 mg/l produced higher number of shoots per shoot tip but were shorter than those produced in 0.1 mg/l BAP and 0.05 mg/l NAA shoots on the same explant share nutrients absorbed by the explant and the more the shoots, the higher the competition of the nutrients. There could have been less competition among the shoots in 0.1 mg/l BAP and 0.05 mg/l NAA leading to tall shoots.Pre-treatment of shoots in 2.5 mg/l IBA followed by culture on medium with vermiculite, was the most suitable for rooting of shoots derived from shoot tip explants of the three papaya lines. Panjaitan et al. (2007) reported that exposure of shoots to 1.0 mg/l IBA for 1 week followed by a transfer to a medium supplemented with vermiculite stimulated 90% of the shoots to produce roots.

The difference between the present results and those of Panjaitan et al. (2007) may be related to the genotype of the plant used. Good aeration in vermiculite must have contributed greatly to root development. Lai et al. (1998) observed that when papaya shoots were rooted in vermiculite under aerated conditions, the vessel ventilation was good, and no ethylene was detected and this may be the reason that individual shoots cultured in the aerated vermiculite medium for root development had vigorous growth and developed healthy roots.

Thus, a commercially viable protocol for micropropagation of selected papaya lines was developed. The shoot multiplication was optimal in MS media supplemented with 0.5 mg/l BAP and 0.1 mg/l NAA, shoot elongation in 0.1 mg/l BAP and 0.05 mg/l NAA and rooting in 2.5 mg/l IBA.

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Seed yield and quality of pepper plants grown under salt stress

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The effect of salinity on seed yield and quality of pepper plants were evaluated. Plants were grown in five salt levels (electrical conductivity, EC): 1.0, 1.5, 2.0, 4.0 to 6.0 dSm^{-1} in glasshouse. Seed yield was assessed by seed weight/fruit, seed weight/plant and individual seed weight. Seed quality was measured by germination, mean germination time, seedling growth and germination percentages after cold (10°C, 7 days, 25°C 10 days) accelerated ageing (45°C, 48 h) and high temperature germination (35°C, 14 days) tests. Results indicate that seed yield per fruit greatly reduced from 1990 mg at control to 460 mg at 4 dSm^{-1} but individual seed weight did not change until 1.5 dSm^{-1} EC. Seed quality concerning all criteria maintained high up to 2.0 dSm^{-1} EC while seed germination and vigour were significantly reduced at 4.0 dSm^{-1} EC. Pepper plants did not yield seeds at 6 dSm^{-1} EC. It can be concluded that pepper seed yield reduced significantly at even very low salinity (1 dSm^{-1}), however, quality began to decline after 2.0 dSm^{-1} EC.

Key words: Pepper, fruit yield, seed quality, salinity.

INTRODUCTION

Salinity is one of the basic abiotic stresses particularly effective in arid and semi-arid regions (Maas, 1986). There are large number of studies that deal with the effects of salinity on germination, seedling growth, plant growth and fruit yield in various crops (Maas, 1986; Cayuela et al., 2001; Shannon and Grieve, 1999). Pepper was considered to be moderately sensitive crop and, regarding fruit yield, threshold electrical conductivity level was reported to be 2.0 dSm^{-1} (Maas, 1986). However, this may not necessarily be applicable for seed yield and quality because, seed maturation is a reproductive stage of the pepper plant and requires longer time than that of fruit on the mother plant. Demir and Ellis (1992) reported that pepper fruits reached full marketable size after 30 to 35 days, while maximum seed quality was obtained from fruits harvested 70 days after flowering. Although numerous studies were conducted on the effect of salinity on plant growth and fruit yield, very little is known about the effect of saline environments on seed quality (Maas, 1986; Shannon and Grieve, 1999). In this work, we aimed to determine changes in yield and quality of pepper seeds obtained from plants grown under various salinity levels in greenhouse conditions.

MATERIALS AND METHODS

Seeds were sown in seedling trays then, transplants were transferred to the pots. Plants (*Capsicum annuum* L. cv. Carliston) were grown in plastic pots which were 50 cm in depth and 40 cm diameter in greenhouse conditions in April and September 2006. Each pot was filled withwashed and dried 9 kg of garden soil (43 % clay, 33 % sand and 24 % silt).

Five salinity levels, made by diluting NaCl with tap water and control were used. The electrical conductivity (EC) of the prepared saline water was 1, 1.5, 2.0, 4.0 and 6.0 dSm^{-1} while EC of the

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Table 1. Fruit (fruit number/plant F/P, average fruit weight AFWT (g)) and seed yield (Seed weight/fruit SWT/F (mg), seed weight /plant SWT/P (mg), individual seed weight ISWT (mg)) of pepper plants grown at various salinity levels.

NaCl (dSm-1)	F/P	AFWT (g)	SWT/F(mg)	SWT/P (mg)	ISWT(mg)
0	4.0^{ab}	118^{b}	497^{a}	1990^{a}	6.2^{a}
1.0	5.3^{a}	142^{a}	187^{bcd}	992^{b}	6.0^{a}
1.5	4.1^{ab}	134^{a}	181^{cd}	745^{c}	6.1^{a}
2.0	3.0^{bc}	104^{c}	180^{cd}	542^{d}	5.6^{a}
4.0	2.7^{c}	74^{d}	170^{e}	460^{e}	3.8^{b}
6.0	NA	NA	NA	NA	NA

NA, No seeds were obtained, means with different letters in the same column is significantly different (P=0.05).

control was 0.25 dSm^{-1}. To provide salinity levels 0.09 g/l NaCl, 0.55 g/l $CaCl_2$ for 1 dSm^{-1}, 0.11 g/l NaCl, 0.773 g/l$CaCl_2$ for 1.5 dSm^{-1}, 0.126 g/l NaCl, 1.49 g/l $CaCl_2$ for 2 dSm^{-1}, 0.18 g/l NaCl, 3 g/l $CaCl_2$ for 4 dSm^{-1}, 0.23 NaCl, 4.89 $CaCl_2$ for 6 dSm^{-1} were dissolved in a litter of non-saline water. Specific absorption rate (SAR) values of irrigation water at all salinity level was lower than 1. Plant nutrition needs were met with 0.733 g K/pot and 0.746 g P/pot at the time of planting. N requirement was supplied by adding 1.76 g/pot at planting and flowering stages, respectively. Control plants were irrigated with non-saline water and treated plants with appropriate EC solutions from the time that plants had five true leaves. Soil moisture was observed in 2-3 day intervals by means of weighing the pots. Irrigation was done when 50% of available soil water was extracted. Daily mean maxima and minima in the greenhouse were 38±2 and 21±3°C, respectively. Six plants were grown in each concentration.

At the end of September, fruit number/plant and fruit weight (g) were determined. Seeds were extracted from fruits at the day of harvest (first and second layer of the plants, 65-70 days after anthesis) and dried at 20±2°C for 24 h (10 % moisture content) then, seed weight/fruit (mg), seed weight/plant (mg) and individual seed weight(mg) were determined in six plants of each concentration. All seeds obtained from the plants grown in the same concentration were mixed up and seed quality tests were carried out. Plants that were watered with 6 $dScm^{-1}$ EC were shrivelled and fruits were severely malformed and seeds were not obtained. Germination of seeds were carried out in Petri dishes (9 cm diameter) containing two Whatman (No:1) filter paper imbibed with 5 ml of distilled water. Three replicates of 50 seeds were germinated in each seed lot. Seeds were allowed to germinate at 25°C in the dark for 14 days. Radicle protrusion (2 mm), normal seedling (firm, well developed hipocotyl and root structure), and abnormal seedling (deformed seedlings) were determined. Mean germination time was calculated according to Ellis and Roberts (1980) on the basis of daily counts and expressed as hours.

At the end of the germination test, normal seedlings were separated in each of three replicates of each concentration and seedling fresh (mg/plant) and dry weight (mg/plant) were determined. Dry weight was determined after 24 h at 80°C. Root length measurements were conducted on seedlings that germinated at 7th day of germination test and expressed as cm/plant. Cold test was carried out on each lot three replications of 50 seeds were sown 2 cm deep in compost in sandwich boxes (18x10x5 cm) and wetted with 50 ml water. Sandwich boxes with lid on were kept at 10°C for 7 days in the dark. They were then transferred to 25°C and normal seedlings that appeared at the surface were counted after 10 days. Accelerated ageing test was carried out on three replicates of 50 seeds in each lot. Forty ml of distilled water added at the bottom of plexiglass box (11x 11x 4 cm). Seeds were placed on wire mesh trays approximately 2 cm above the bottom of the tray and aged at 45°C for 48 h. Following this period, the seeds were removed and a germination test (radicle protrusion) conducted as described above.

High temperature germination test in each lot was conducted on three replicates of 50 seeds at 35°C. Radicle protrusion was counted after 14 days. Means of the replicates of the each test in each concentration were compared with Duncan multiple range test. Germination percentage was angle transformed before analyses.

RESULTS

Fruit weight, fruit number, seed weight per plant and fruit and individual seed weight are presented in Table 1. This study indicates that pepper seed yield in either fruit or plant was gradually declined as salinity increased. The maximum reduction occurred at 4 dSm^{-1} EC compared to the control at 51%. Individual seed weight did not change up to 2.0 dSm^{-1} EC (Table 1). Seeds that were obtained from plants grown at 1.0, 1.5 and 2.0 dSm^{-1} EC salinity had as high quality (Normal germination, cold test, accelerated ageing test, high temperature germination test) as those of the control. However, the lowest seed quality was obtained from plants that were irrigated with water of 4.0 dSm^{-1} EC (Tables 2 and 3).

Seedling dry weight and root length were not significantly changed in seeds produced up to 2.0 dSm^{-1} EC but both declined greatly by 4.0 dSm^{-1} and being 1.3 mg/plant and 1.9 cm, respectively (Table 2). Maximum seedling fresh weight was recorded in between 1 and 2.0 dSm^{-1} EC as 28 to 31 mg/plant. Seedling fresh weight of both seeds of control and at 4.0 dSm^{-1} EC were significantly lower than these three lots and found as 25 and 20 mg/plant, respectively (Table 2). Cold test results show that seed germination is not significantly affected by irrigating plants with saline water up to 2.0 dSm^{-1} EC while, slightly higher salinity (1-2.0 dSm^{-1}) increased germination percentages after ageing test compared to that of control. Similar effect was also observed in high temperature germination test. The lowest values were obtained from those seeds grown at 4.0 dSm^{-1} EC as 25,

Table 2 Changes in the radicle protrusion (RP, %), normal and abnormal germination (NG and AG %) and mean germination time (MGT h), seedling fresh (SFW, mg/plant), seedling dry weight (SDW, mg/plant) and root length (RL, cm/plant) of pepper seeds that obtained from plants grown at various salinity levels.

NaCl (dSm^{-1})	RP (%)	NG (%)	AG (%)	MGT (h)	SFW (mg/plant)	SDW (mg/plant)	RL (cm/plant)
0	97^{a}	71^{b}	26^{b}	91^{e}	25^{b}	2.2^{a}	2.6^{a}
1.0	97^{a}	79^{a}	18^{c}	93^{de}	28^{ab}	2.2^{a}	2.8^{a}
1.5	91^{a}	78^{ab}	13^{d}	100^{c}	30^{a}	2.3^{a}	2.6^{a}
2.0	95^{a}	81^{a}	14^{d}	103^{bc}	31^{a}	2.3^{a}	2.7^{a}
4.0	82^{a}	40^{c}	42^{a}	118^{a}	20^{c}	1.3^{b}	1.9^{b}
6.0	NA	NA	NA	NA	NA	NA	NA

NA, No seeds were obtained; means with different letters in the same column is significantly different (P=0.05).

Table 3. Changes in germination percentage of pepper seeds after cold, (CT, %) accelerated ageing (AAT, %) and high temperature germination (HTGT, %) tests that were obtained from plants grown at various salinity levels.

NaCl (dSm^{-1})	CT (%)	AAT (%)	HTGT (%)
0	42^{a}	63^{c}	61^{b}
1.0	43^{a}	72^{b}	76^{a}
1.5	39^{a}	79^{a}	71^{a}
2.0	43^{a}	76^{ab}	70^{a}
4.0	25^{b}	32^{d}	34^{c}
6.0	NA	NA	NA

NA, No seeds were obtained; means with different letters in the same column is significantly different (P=0.05).

32 and 34%, for cold, accelerated ageing and high temperature germination test, respectively (Table 3).

DISCUSSION

Large number of studies in various vegetable crops investigated the effect of salinity on vegetative plant development stages such as germination and seedling growth, plant shoot and root dry weight and yield (Maas, 1986; Shannon and Grieve, 1999; Demir et al., 2003; Santamaria et al., 2004). Maas (1986) classified pepper as a moderately sensitive crop and maximum permissible concentration in soil water without yield reduction was given as 1.0-2.0 dSm^{-1} EC. Concerning fruit yield, the present results are in agreement with this finding. However, seed yield declined greatly even at 1 dSm^{-1} EC compared to those of the control plants (Table 1). Therefore, it can be concluded that the level of salt tolerance based on vegetative plant growth stages or fruit yield may not necessarily be a good indicator of reproductive criteria of seed yield and quality in pepper. Reduction in seed yield and quality preceded that of fruit yield under salt stress. This may be due to limitation in assimilate allocation to seeds by salinity. The reduction of the individual mass of seeds under high salinity levels has also been observed in wheat (Saini and Aspinall, 1981).

Salt injury symptoms, such as chlorosis, burning leaves, and necrotic areas were found severely in plants grown at 6 dSm^{-1} EC and no seeds were obtained from that concentration. Salinity stress during plant development might cause scorching, firing of leaves, shorter stature which cause slow seed maturation rate (Wahid et al., 1999). Reduced seed yield and quality at higher salinity levels in this work might originate from physiological occurrences that reduce dry matter accumulation and partitioning (Fenner, 1992; Dkhil and Denden, 2010). Increase in fruit number/ plant and average fruit weight were observed at 1 and 1.5 dSm^{-1} EC compared to those of the control (Table 1). This is in agreement with conclusions of some earlier reports which showed that moderate salinity applied during fruit development changed the partitioning of photosynthesis and improve soluble solids in melon and tomato fruits (Shannon and Grieve, 1999; Mizrahi et al. 1988). This agrees with findings of the previous reports which showed that moderate salinity applied during fruit development improves not only soluble solids but also seed vigour (Shannon and Grieve, 1999).

Pepper is a warm climate crop and requires relatively high temperatures, that is, 27 to 30°C, for optimum crop growing (George, 1985). In glasshouse pepper seed crop growing, temperatures in summer months raises up to 40 to 45°C which exacerbates the effect of soil salinity. Such high temperatures are common phenomenon in

Mediterranean region and accelerate salt stress due to increase evaporation and the remaining soil water becomes more concentrated. However, the present results are valuable for seed producers who wish to produce pepper seeds in moderately saline areas. But further work is required in order to determine the interactive effect of environmental factors (that is, temperature, soil type) with salt tolerance in pepper seed crop.

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Full Length Research Paper

Physiological studies of *Sclerotium rolfsii* Sacc. causing collar rot of peppermint

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***In vitro* studies were conducted on the effect of temperature, pH levels, carbon, nitrogen and amino acids on the mycelial growth and biomass production of *Sclerotium rofsii* Sacc. causing collar rot of mint. The results reveal that the growth of *S. rolfsii* was maximum at 30°C which was reduced significantly below 20°C and above 35°C. Of the pH levels tested, acetic pH (5.0) produced maximum mycelial dry weight which was followed by exposing the pathogen to pH 6.0. Among the nine carbon sources tested, sucrose recorded the maximum mycelial growth and dry weight of *S. rolfsii*, while peptone was the best among the nitrogen sources and tryptophane and phenylalanine was the best amino acids on the mycelial growth and biomass production of *S. rolfsii*.**

Key words: *Sclerotium rolfsii*, pH, mint, collar rot, nutritional studies, temperature.

INTRODUCTION

Peppermint (*Mentha piperita* L.) is an important aromatic perennial herb grown throughout the world, belonging to the family Lamiaceae. It is extensively cultivated in India and about 70% of the International annual requirement is met from crops raised in the central region of the Indo-Gangetic plains (Singh et al., 1999). *Mentha* is cultivated in Himalaya-hills, Haryana, Uttar Pradesh, Punjab and Bihar. Of these, Uttar Pradesh is the largest producing state in the country contributing 80 to 90% of the total production followed by Punjab, Haryana, Bihar and Himachal Pradesh. Cultivated peppermint mint, serves as a source of menthol, menthone, isomenthone, mentho-furan, linanool, linalyl acetate, methyl acetate, terpenes, carvone, piperitenone oxide and other aromatic com-pounds.

In India, peppermint is grown throughout the year (Shukla et al., 1998) and it is affected by several fungal diseases; of which, collar rot caused by *Sclerotium rolfsii* is a major constraint in the peppermint cultivation in Tamil Nadu. *S. rolfsii* is a soil borne plant pathogen causing root rot, stem rot, collar rot, wilt and foot rot diseases on more than 500 plant species of agricultural and horti-cultural crops throughout the world (Aycock, 1966). The pathogen causes a great economic loss on various crops. It has been reported that *S. rolfsii* caused about 25% seedling mortality in the groundnut cultivar JL-24 (Ingale and Mayee, 1986). In tomato, this pathogen was respon-sible for a crop loss of 30% (Thiribhuvanamala et al., 1999). Its occurrence on crossandra has been observed about 40 to 50% mortality of plants.

In peppermint, this pathogen caused about 5 to 20% of crop loss was observed under field condition (Anand and Harikesh Bahadur, 2004). These pathogens exhibit varia-tion in their morphological biological and immunological characteristics and pathogenicity or resistance against harmful environment. Morphogenic and pathogenic varia-tions are known in many fungal pathogens and as such detailed investigation was carried out on the variations with regards to pH, temperature, nutritional factors on the mycelia growth and biomass production of *S. rolfsii*.

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MATERIALS AND METHODS

Isolation, identification and maintenance of pathogen

The collar rot symptoms were collected from the Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Chidambaram, Tamil Nadu, India. The infected plant materials brought back from the field were washed, cut into 5 mm segments including the advancing margins of infection. The segments were surface sterilized in 0.5% sodium hypochlorite solution for 5 min and rinsed in three changes of sterile distilled water. The segments were separately dried in between sheets of sterile filter paper and placed (3 pieces per plate) on fresh Potato Dextrose Agar (PDA) medium (Ainsworth, 1961) impregnated with streptomycin, and incubated for seven days at 28 ± 2°C.

The fungal growth on 5th day, which arose through the sclerotial bodies was cut by inoculation loop and transferred aseptically to the PDA slants and allowed to grow at room (28 ± 2°C) temperature to obtain the pure culture of the fungus.

The culture thus obtained was stored in refrigerator at 5°C for further studies and was sub cultured periodically. The purified isolate was identified based on morphological and colony characteristics (Punja and Damini, 1996; Sarma et al., 2002; Watanabe, 2002).

Effect of different temperature levels on the mycelial growth of *S. rolfsii in vitro*

Solid medium

A quantity of 15 ml of the sterilized potato dextrose agar medium was poured into 90 mm sterile Petri plates. The plates were inoculated with 6 mm mycelial disc of the pathogen obtained from 7 days old grown on PDA and incubated in BOD at different temperature (5, 10, 15, 20, 25, 30 and 35°C) for 7 days in an incubator. The mycelial growth of the pathogen was measured in mm at the end of incubation period.

Liquid medium

Erlenmeyer flasks (250 ml) containing 50 ml of potato dextrose broth were sterilized, inoculated and incubated at different temperatures namely: 5, 10, 15, 20, 25, 30 and 35°C for 10 days in BOD incubator.

At the end of the incubation period the mycelial mat was filtered through Whatman No. 41 filter paper of known weight. The filter paper with mycelial mat was dried in hot air oven at 105°C for 48 h and the mycelial dry weight was calculated. In both the methods, three replications were maintained for each treatment.

Effect of certain pH levels on the mycelial dry weight of *S. rolfsii in vitro*

Potato dextrose broth of different pH levels (1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0) were prepared and sterilized. They were inoculated with 6 mm mycelial disc of the pathogen obtained from 7 days old grown on PDA.

The flasks were incubated for 10 days at 28 ± 2°C in BOD incubator. After incubation, the fungal biomass was separated through filtration in a previously dried and weighed filter paper (Whatman No. 41). Then the mycelial dry weight was calculated. Three replications were maintained for each treatment.

Effect of certain nutritional factors on the mycelial growth of *S. rolfsii in vitro*

Linear growth of S. rolfsii on solid medium

Czapek's agar medium was amended with various carbon sources, nitrogen sources and amino acids on equivalent weight basis and were dispensed in sterile Petri plate at 15 ml quantities. After cooling, they were inoculated with 6 mm mycelial disc of the pathogen obtained from 7 days old culture grown on PDA in Petri plates and incubated for 7 days. The linear growth of the pathogen was measured in mm at the end of the incubation period.

Carbon source

Growth on liquid medium

The *in vitro* growth of the fungus was tested with nine different carbon sources (cellulose, dextrose, fructose, glucose, lactose, maltose, mannitol, starch and sucrose). Czapek's broth was taken as the basal medium for the study. In the czapek's broth, sucrose was replaced with various carbon sources on equivalent weight basis and autoclaved at 15 lb pressure for 20 min.

Nitrogen source

Growth on liquid medium

The *in vitro* growth of the fungus was tested with nine different nitrogen sources (ammonium chloride, ammonium nitrate, ammonium sulphate, calcium nitrate, peptone, potassium nitrate, sodium nitrite, sodium nitrate and urea). Czapek's broth was taken as the basal medium for the study. In the czapek's broth, sodium nitrate was replaced with various nitrogen sources on equivalent weight basis and autoclaved at 15 lb pressure for 20 min.

Amino acids

Growth on liquid medium

The *in vitro* growth of the fungus was tested with eight different sources of amino acids namely: alanine, asparagine, cysteine, glutamic acid, phenylalanine, tryptophan, tyrosine and valine. Czapek's broth was taken as the basal medium for the study. In the czapek's broth, the nitrogen source was replaced with various amino acids on equivalent weight basis and autoclaved at 15 lb pressure for 20 min. In all the cases, the final pH of the medium was adjusted to 7.0.

After that, the medium was inoculated with 6 mm mycelial disc of pathogen obtained from 7 days old culture grown on PDA. The inoculated media were incubated for 10 days at room temperature (28 ± 2°C). At the end of the incubation period, the mycelial mats were filtered through previously dried and weighed filter paper (Whatman No. 41) and dried in hot air oven at 105°C for 48 h and the mycelial dry weight was calculated. Three replications were maintained for each treatment.

RESULTS AND DISCUSSION

Effect of different temperature levels

Among the temperature levels (6, 10, 15, 20, 25, 30 and 35°C) tested, 30°C was found to be more conducive for the mycelial growth of *S. rolfsii* (89.00 mm) under *in vitro* recording the highest mycelial dry weight of 610.66 mg, which was followed by 25° (Figures 1 and 2). However, the exposure of *S. rolfsii* to high temperature that is, 35°C was found to be highly detrimental to the growth of *S. rolfsii*. Each pathogen has got its own cardinal temperature and understanding the temperature requirement of the pathogen will help to standardize the management

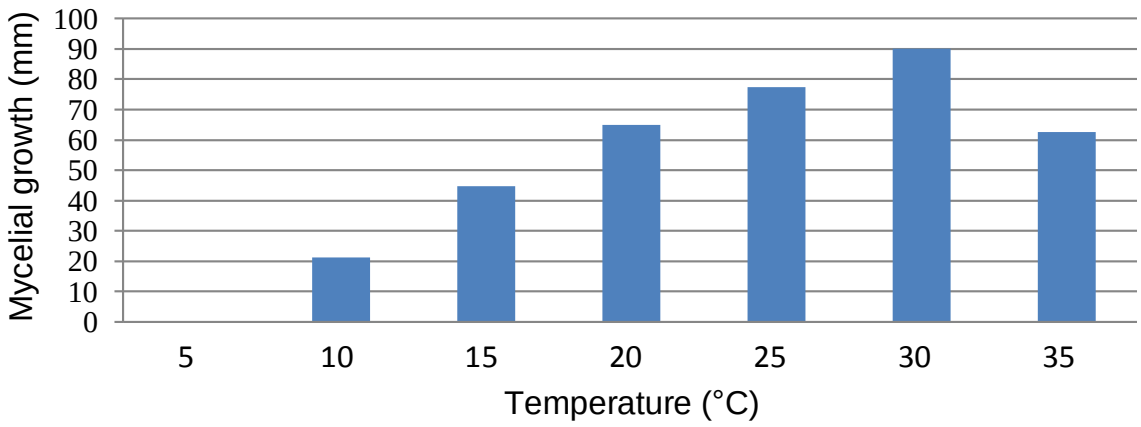

Figure 1. Effect of different temperature levels on the mycelial growth of *Sclerotium rofsii*.

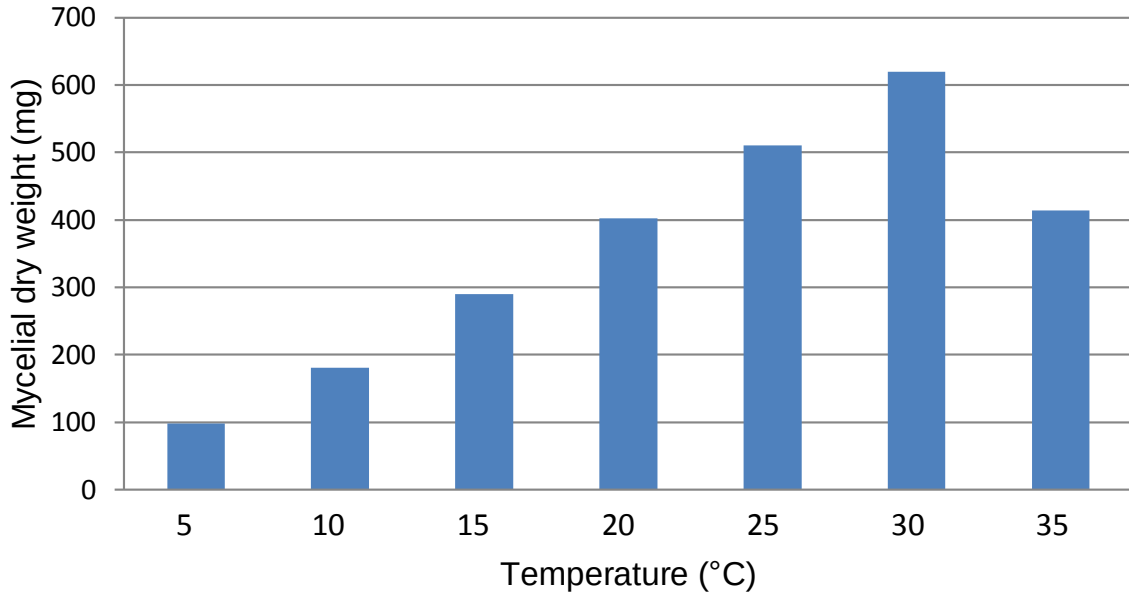

Figure 2. Effect of different temperature levels on the mycelial dry weight of *Sclerotium rolfsii*.

practices. It plays an important role in the growth and reproduction of fungi. Earlier published reports also clearly indicated that the optimum temperature for the *in vitro* growth of *S. rolfsii* is in the range of 25 to 30°C. Mahen et al. (1995), Tripathi and Khare (2006), Basamma (2008) and Lin et al. (2009) reported that 25 to 30°C was more conducive for the vegetative growth of *S. rolfsii*. Recently, Zape et al. (2013) reported that the *S. rolfsii* showed rapid mycelial growth at 30°C and maximum sclerotial production was recorded at 25°C.

Effect of different pH levels

The results presented in Figure 3 showed that among the pH levels (1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0) tested, exposure of pathogen to acetic pH (5.0) produced maximum mycelial dry weight recording 514.66 mg which was followed by exposing the pathogen to pH 6.0 under *in vitro*. Increase (or) decrease in pH beyond 5.0 and 6.0 was not conducive for the growth of pathogen.

This finding was inline with the earlier reports made by Dey et al. (1992), Mahen et al. (1995) and Basamma (2008). Recently, Zape et al. (2013) reported that the optimum pH for the mycelial growth and sclerotial formation of *S. rolfsii* was pH 5.5 to 7.5. The fungi generally utilize substrate in the form of solution only if the reaction of the solution is conductive to fungal growth and metabolism. This brings the importance of hydrogen ion concentration for the better fungal growth. In general, the pathogen showed preference for pH level towards acidic side.

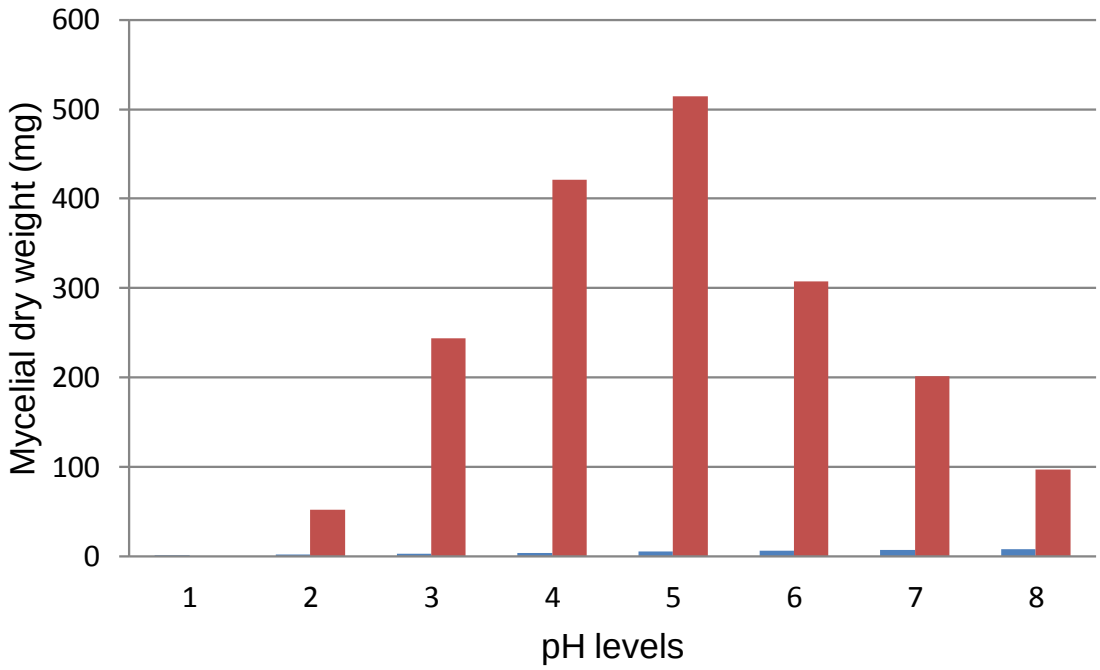

Figure 3. Effect of different pH levels on the mycelial dry weight of *Scelrotium rolfsii.*

Table 1. Effect of different carbon sources on the mycelial growth and dry weight of *S. rolfsii.*

Carbon source	Mycelial growth (mm)	Mycelial dry weight (mg)
Cellulose	50.66[e]*	210.00[f]*
Dextrose	80.66[b]	610.00[b]
Fructose	63.66[d]	250.00[e]
Glucose	75.00[c]	533.66[c]
Lactose	45.66[g]	100.66[h]
Maltose	53.00[e]	230.33[f]
Mannitol	47.00[f]	170.33[g]
Starch	74.33[c]	495.66[d]
Sucrose	89.33[a]	650.66[a]
Control	52.00[e]	70.33[i]

* Value within a column with same letter do not differ significantly according to the DMRT method (P = 0.05).

Effect of different carbon sources

Among the nine carbon sources tested, sucrose recorded the maximum mycelial growth of 89.33 mm and dry weight of 650.66 mg (Table 1). It was succeeded by dextrose and glucose. However, mycelial growth and dry weight was minimum with lactose as carbon source. This was similar to the findings of Prasad et al. (1986), Chun et al. (2003), Survase et al. (2006) and Xiao et al. (2012). Almost half of the mycelial dry weight of the fungal cell consists of carbon which is the main structural element (Lilly and Barnett, 1951). The utilization of various carbon compounds may depend on either of the activity of the fungus to utilize simpler forms or on its power to convert the complex carbon compounds into simpler forms, which may be easily utilized. Sucrose is generally utilized as a good source by most of the plant pathogenic fungi.

Effect of different nitrogen sources

The data presented in Table 2 revealed that all the nitrogen sources namely, ammonium chloride, ammonium nitrate, ammonium sulphate, calcium nitrate, peptone, potassium nitrate, sodium nitrite, sodium nitrate and urea favoured the growth of *S. rolfsii.* Among the nine nitrogen sources tested, peptone recorded maximum mycelial growth (89.66 mm) and dry weight (790.33 mg) of *S. rolfsii*. Culturing of *S. rolfsii* in calcium nitrate and ammonium

Table 2. Effect of different nitrogen sources on the mycelial growth and dry weight of *S. rolfsii.*

Nitrogen source	Mycelial growth (mm)	Mycelial dry weight (mg)
Ammonium chloride	67.33[e]*	410.00[g]*
Ammonium nitrate	73.66[d]	620.33[e]
Ammonium sulphate	70.00[d]	590.66[f]
Calcium nitrate	65.33[e]	350.00[h]
Peptone	89.66[a]	790.33[a]
Potassium nitrate	85.00[b]	750.00[b]
Sodium nitrite	84.33[b]	730.66[c]
Sodium nitrate	80.00[c]	690.66[d]
Urea	82.00[c]	700.00[d]
Control	52.66[f]	71.66[i]

* Value within a column with same letter do not differ significantly according to the DMRT method (P = 0.05).

Table 3. Effect of different amino acids on the mycelial growth and dry weight of *S. rolfsii.*

Amino acid	Mycelial growth (mm)	Mycelial dry weight (mg)
Alanine	70.00c*	600.00d*
Asparagine	67.33d	520.33e
Cysteine	72.66c	670.00c
Glutamic acid	65.00d	405.66f
Phenylalanine	89.33a	785.00a
Tryptophan	89.66a	780.66a
Tyrosine	60.00e	320.00g
Valine	77.33b	698.33b
Control	53.00f	72.00h

* Value within a column with same letter do not differ significantly according to the DMRT method (P = 0.05).

chloride was found to be recording minimum mycelial growth and dry weight of *S. rolfsii*. Similarly, Azhar Hussain et al. (2003), Khattabia et al. (2004) and Basamma (2008) reported that potassium nitrate and peptone recorded the maximum mycelial growth of *S. rolfsii*. Nitrogen being a component of protein is an essential element and like carbon, it is used by fungi for functional as well as structural purposes. But all the sources of nitrogen are not equally good for the growth of fungi.

Effect of various amino acids

The effect of various amino acids on the mycelial growth and dry weight of *S. rolfsii* are summarized in Table 3. Among the eight amino acids (alanine, asparagine, cysteine, glutamic acid, phenylalanine, tryptophan, tyrosine and valine) tested, phenylalanine and tryptophane supported maximum mycelial growth and dry weight of *S. rolfsii* when compared to control which was followed by valine. The minimum mycelial growth and dry weight was registered in tyrosine. Similarly, Muthukumar and Eswaran (2008a) who explained that maximum mycelial growth and dry weight of *Pythium aphanidermatum* was recorded in phenylalanine and tryptophan amended medium. So far no report is available on this area. A large number of amino acids are recorded as good nutritional sources for many fungi. However, the nutritive capacity of individual amino acids varies highly with organisms.

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Full Length Research Paper

Effect of light and aeration on the growth of *Sclerotium rolfsii in vitro*

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Sclerotium rolfsii **is one of the devastating soil-borne plant pathogens which cause severe loss at the time of seedling development. It also causes leaf spots in several crops and wild plants. In this experiment, exposure of pathogen to different light period and aeration in order to assess the mycelial growth, biomass production, weight and number of sclerotia of *S. rolfsii* was done. Three-fourth area of three plates, 50% area of three plates and 100% area of three plates were sealed with cellophane tape. The other three plates were not sealed. Two sets of such plates were prepared. All the plates were incubated at 28±2°C. One set was incubated in light whereas the other set was incubated in the dark. The results reveal that there was no significant difference in mycelial growth and number of sclerotia among them but significant difference was observed when compared with the control, that is, the plates which were not sealed. Sclerotial formations were directly influenced by air as completely sealed plates failed to produce sclerotia. Generally, the light condition induces the production of more number of sclerotia than dark condition. In another study, the exposure of pathogen to different light periods revealed that alternative cycles of 12 h light and 12 h darkness for ten days resulted in the maximum mycelial growth and dry weight, more number of sclerotia and weight of sclerotia was also seen when compared with other treatments.**

Key words: *Sclerotium rolfsii*, aeration, light, peppermint.

INTRODUCTION

Peppermint (*Mentha piperita L.*) is an important aromatic perennial herb grown throughout the world; it belongs to the family Lamiaceae. It is extensively cultivated in India and about 70% of the international annual requirement is met from crops raised in the central region of the Indo-Gangetic plains (Singh et al., 1999). *Mentha* is cultivated in Himalaya-hills, Haryana, Uttar Pradesh, Punjab and Bihar. Of these, Uttar Pradesh is the largest producing state in the country contributing 80-90% of the total production followed by Punjab, Haryana, Bihar and Himachal Pradesh. Cultivated peppermint, serves as a source of menthol, menthone, isomenthone, menthofuran, linanool, linalyl acetate, methyl acetate, terpenes, carvone, piperitenone oxide and other aromatic compounds. In India, peppermint is grown throughout the year (Shukla et al., 1998) and it is affected by several fungal diseases caused by *Rhizoctonia solani* (Kumar et al., 1997; Merin, 2002), *Verticillium dahliae* (Johnson and Santo, 2001), *Collectotrichum cocodes* (Johnson et al., 2002) and *Sclerotium rolfsii* (Anand and Harikesh Bahadur Bahadur, 2004) of which, collar rot caused by *S. rolfsii* is a major constraint in the peppermint cultivation in Tamil Nadu. *S. rolfsii* is a soil borne plant pathogen causing root rot, stem rot, collar rot, wilt and foot rot diseases on more than 500 plant species of agricultural and horticultural crops throughout the world (Aycock, 1966). The pathogen causes

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a great economic loss in various crops. It has been reported that *S. rolfsii* caused about 25% seedling mortality in the groundnut cultivar JL-24 (Ingale and Mayee, 1986). In tomato, this pathogen was responsible for a crop loss of 30% (Thiribhuvanamala et al., 1999). Its occurrence on crossandra has been observed to be about 40-50% mortality of plants. In peppermint, this pathogen caused about 5 to 20% of crop loss under field condition (Anand and Harikesh, 2004). Diseases caused by *S. rolfsii* are initiated either directly from soil-borne sclerotia which germinate to form fine cottony hyphae infecting the collar region of host plants or sclerotia sticking on the lower/upper surfaces of the leaves by rain splashes where they germinate and cause leaf spots (Singh and Pavgi, 1965). Soil temperature of 25-30°C and soil moisture 90% play significant role in disease development (Gupta et al., 2002). Various biotic and abiotic factors which directly or indirectly influence the development of sclerotia were discussed in literature (Ellil, 1999; Sarma, 2002). The objectives of the present study were i) to isolate and identify the pathogen ii) to study the pathogenicity test iii) to study the role of air in the growth of *S. rolfsii* and influence of light on the growth of pathogen.

MATERIALS AND METHODS

Isolation, identification and maintenance of pathogen

The collar rot symptoms were collected from the Department of Plant Pathology, Faculty of Agriculture, Annamalai University, Chidambaram, Tamil Nadu, India. The infected plant materials brought back from the field were washed, cut into 5 mm segments including the advancing margins of infection. The segments were surface sterilized in 0.5% sodium hypochlorite solution for 5 min. and rinsed in three changes of sterile distilled water. The segments were separately dried in between sheets of sterile filter paper and placed (3 pieces per plate) on fresh potato dextrose agar (PDA) medium (Ainsworth, 1961) impregnated with streptomycin, and incubated for seven days at 28 ± 2°C. The fungal growth on 5^{th} day, which arose through the sclerotial bodies was cut by inoculation loop and transferred aseptically to the PDA slants and allowed to grow at room (28 ± 2°C) temperature to obtain the pure culture of the fungus. The culture thus obtained was stored in refrigerator at 5°C for further studies and was sub cultured periodically. The purified isolate was identified as *S. rolfsii* based on morphological and colony characteristics (Punja and Damini, 1996; Sarma et al., 2002; Watanabe, 2002).

Assessing the pathogenicity of *S. rolfsii* isolate

The pot mixture was prepared by thoroughly mixing clay loam soil, sand and farm yard manure at the ratio of 1:1:1. The inoculum of *S. rolfsii* isolate was grown on sand-corn meal medium (twenty days old) mixed thoroughly at five percent (w/w basis) level, and applied to top two centimeter of the soil (Abeygunawardena and Wood, 1957). Then, apparently healthy surface sterilized mint cuttings were planted in inoculated pots. The cuttings planted in pots without inoculum served as control. Soil moisture was maintained at moisture holding capacity of soil by adding sterilized water on weight basis throughout the period. After 20 days of inoculation, the plants showing the typical wilting symptoms were observed. Re-isolation was made from such affected portion of the plant tissue and compared with that of original isolate for conformity.

Effect of air on sclerotial development of *S. rolfsii* in potato dextrose agar medium

Fifteen milliliters of molten PDA medium was dispensed into 12 sterile Petri plate. Mycelial discs taken from the advancing margins of seven days old culture of respective *S. rolfsii* isolate by the aid of cork borer were separately placed at the centre of the plate containing PDA medium. Three-fourth area of three plates, 50% area of three plates and 100% area of three plates were sealed with cellophane tape. The other three plates were not sealed. Two sets of such plates were prepared. All the plates were incubated at 28±2°C. One set was incubated in light whereas the other set was incubated in the dark. In this experiment, there were four treatments and each treatment consists of three plates and each treatment is repeated three times. The inoculated plates were sealed with the help of lab seal in the following manner, that is, no sealing (control), half sealed, $3/4^{th}$ and complete sealing. Each set contained three plates. After inoculation and sealing, Petri plates were incubated at 28 ± 2°C (light and dark) and the other sealed plates were wrapped with black paper and incubated as above. Visual observations were periodically made for sclerotial initiation, sclerotial development and number of sclerotia per plate.

Effect of light on the growth of *S. rolfsii*

Potato dextrose broth and agar were used in this experiment. Conical flasks of 250 ml capacity and each containing 100 ml of liquid broth were inoculated and exposed to different length of light hours viz., alternate cycles of twelve hours light and twelve hours darkness, continuous light and continuous darkness in an environmental conditions. Flasks were inoculated with 6 mm mycelial disc obtained from the periphery of seven days old culture of *S. rolfsii* and incubated at different light intensities. All the inoculated plates were incubated for ten days under different length of light hours. The number of sclerotia/flask and weight of sclerotia was recorded at the end of the incubation period. There were three treatments and each treatment consists of three plates and each treatment was repeated three times. Then the mycelial mat was filtered through Whatman No. 41 filter paper discs of 12.50 cm diameter dried to a constant weight at 60°C prior to filtration. The mycelial mat on the filter paper was washed thoroughly with distilled water to remove any salts likely to be associated with the mycelium and dried to a constant weight in an electrical oven at 60°C, cooled in a dessicator and weighed immediately on an analytical electrical balance. The weight of dry mycelium was recorded and the data were statistically analyzed.

To carryout study on solid media, 15 ml of potato dextrose agar was poured in 90 mm sterile Petri plate. Such plates were inoculated with six mm mycelial disc obtained from the periphery of seven days old culture of *S. rolfsii* and incubated at different light intensities. Each treatment consists of three plates and each treatment was repeated three times. All the inoculated plates were incubated for ten days under different length of light hours. The mycelial growth, number of sclerotia/plate and weight of sclerotia was recorded at the end of the incubation period.

Statistical analysis

The data on effect of the treatments on the growth of pathogens was analyzed by analysis of variance (ANOVA) and treatment means were compared by Duncan's multiple range test (DMRT) and by least significance difference (LSD) at P = 0.05. The package used for analysis was IRRISTAT version 92-1 developed by the

Table 1. Effect of air on sclerotial development of *S. rolfsii* in potato dextrose agar medium.

Treatment	Observation									
	In dark visual observation after (days)				Average number of sclerotia/plate	In light visual observation after (days)				Average number of sclerotia/plate
	6	8	10	12		6	8	10	12	
No sealing (control)	+	++	++	+++	213[a]*	+	++	++	+++	276[a]*
1/2 sealing	+[F]	++	+++	+++	168[b]	+[F]	++	+++	+++	185[b]
3/4 sealing	+[F]	++	+++	+++	157[c]	+[F]	++	+++	+++	178[c]
Complete sealing	-	-	-	-	0[d]	-	-	-	-	0[d]

+- Sclerotial initial; ++- white sclerotia; +[F] -fewer sclerotia initials; +++ - dark brown sclerotia; - = no sclerotial initials; *Values in each column followed by the same letter are not significantly different according to the DMRT method (P = 0.05).

Table 2. Effect of light on the growth of *S. rolfsii* in potato dextrose agar medium and potato dextrose broth.

Treatments	Mycelial growth (mm)	Mycelial dry weight (mg)	Average number of sclerotia/plate	Weight (mg)/100 sclerotia
Continuous light	68.66[b]*	280.33[b]*	225[b]*	74[b]*
Continuous dark	47.33[c]	130.00[c]	156[c]	54[c]
Alternate cycle of 12 h light and 12 h darkness	89.66[a]	382.66[a]	263[a]	85[a]

*Values in each column followed by the same letter are not significantly different according to the DMRT method (P = 0.05).

Biometrics Unit of the International Rice Research Institute, The Philippines (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

The results of the present study reveal that the number of sclerotia in 3/4 and 1/2 sealed plates placed in light and darkness affected the mycelial growth and number of sclerotia significantly as compared to the control (unwrapped plates). In the control plates, sclerotia initials were observed after 6 days of inoculation as whitish, tiny, pinhead-like structures and after 6-8 days exudation commenced. In completely sealed plates, the fungal growth was relatively very slow, compact and profusely growing mycelium was observed after 6 to 8 days as compared to the control. In all completely sealed plates, there was no sclerotium formation even after 12 days after inoculation. In 3/4 and 1/2 sealed plates, the number of sclerotia were less but they were bigger in size as compared to the control. In control plates, mature sclerotia became brownish at 3/4th day after inoculation but in 1/2 and 3/4 sealed plates, such sclerotia were seen after 10 days (Table 1). Sclerotia are the asexual structures formed due to the aggregation of fungal mycelium. Several biotic and abiotic factors influence the aggregation of fungal hyphae in the culture medium. Punja and Damini (1996) and Singh et al. (2002) reported that sclerotial exudates directly influenced the development and maturation of sclerotia. The number and sclerotial weight were affected drastically due to improper aeration as average numbers of sclerotia were more in unsealed plates (Sudarshan et al., 2010). Bhoraniya et al. (2002) reported that due to pathogenesis, the level of oxalic acid increases in the infected plants and the increase of oxalic acid induces formation of sclerotial initiation at the collar region. It was reported that depletion of exudate inhibits the development of sclerotia of *S. sclerotiorum* (Singh et al., unpublished observation).

The exposure of the pathogen to alternative cycles of 12 h light and 12 h darkness for ten days resulted in the maximum mycelial growth and dry weight (89.66 mm; 382.66 mg, respectively) with more number of sclerotia/plate and weight of sclerotia of *S. rolfsii* which was significantly superior over other treatments tested (Table 2). The mycelial growth of pathogen exposed to continuous light resulted in moderate growth (68.66 mm; 280.33 mg) and continuous darkness resulted in minimum mycelial growth and dry weight of *S. rolfsii* (47.33 mm; 130.00 mg) and less number of sclerotia/plate and weight of sclerotia was also very less (156; 54, respectively). Similarly, Basamma (2008) reported that, *S. rolfsii* was exposed to alternate cycles of 12 h light and 12 h darkness recorded more number of sclerotia of *S. rolfsii.* This is in agreement with the findings of Chung and Kim (1977) and Punja (1985).

In the present experiment, we found that a proper aeration and light is essential for the mycelial growth anddevelopment of sclerotia of *S. rolfsii.*

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Production and characterization of antimicrobial active substance from some macroalgae collected from Abu-Qir bay (Alexandria) Egypt

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The antimicrobial activity of three different macroalgal species [*Jania rubens* (Linnaeus) Lamouroux*; Ulva fasciata* Delile and *Sargassum vulgare* C. Agardh] belonging to Rhodophyta, Chlorophyta and Phaeophyceae, respectively, were collected seasonally in 2007 to 2008 from Abu-Qir bay (Alexandria, Egypt). The different macroalgal species were tested against pathogenic microbes such as *Bacillus subtilis, Staphylococcus aureus* and *Streptococcus aureus* as gram-positive bacteria, *Escherichia coli, Salmonella typhi* and *Klebsiella pneumoniae* as gram-negative bacteria and one yeast strain, *Candida albicans*. The influence of sampling season on the antimicrobial activity of the collected seaweeds showed strong activity in spring followed by winter, summer and autumn, respectively. However, the strongest antimicrobial activity was recorded in 70% acetone extract of *U. fasciata* collected during winter against all the tested microorganisms. This extract was purified using column chromatography (CC) and thin layer chromatography (TLC). The nature of this purified antimicrobial material was detected using different chemical analysis (UV, IR, ^{1}H NMR and MS) which indicated that it is an aromatic compound and has different active groups (-NH_2, -C=O, -NO_2, phenyl ring and -CH_3). The molecular weight of the compound was determined (662) and its structure was characterized as a derivative of phthalate ester [(E)-1-(10-acetamido-2-nitrodec-9-enyl) 2-(10-acetamido-2-nitrodecyl) 4-methylphthalate]. This is the first evidence of the isolation of phthalate esters derivative from green seaweeds (*U. fasciata*) that has broad antimicrobial activity.

Key words: Antimicrobial, pathogenic microbes, season, seaweeds.

INTRODUCTION

Infectious diseases are a major cause of morbidity and mortality worldwide (WHO, 2004). The increase in failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity (Colombo and Bosisio 1996; Cordell 2000; Scazzocchio et al., 2001). Synthetic drugs are not only expensive and inadequate for the treatment of diseases, but are also often with adulterations and side effects. Therefore, there is a need to search for new infection-fighting strategies to control microbial infections (Sieradzki and Tomasz, 1999). There are numerous reports of compounds derived from macroalgae with a broad range of biological activities, such as the anti-microbial activities (Reichelt and Borowitzka, 1984; Ballantine et al., 1987; Ballesteros et al., 1992; Vlachos et al., 1996), antiviral diseases (Trono, 1999), antitumors and anti-inflammatories (Scheuer, 1990) as well as neurotoxins (Kobashi, 1989). Subsequent chemical investigations of bioactive extracts led to the discovery of many struc-

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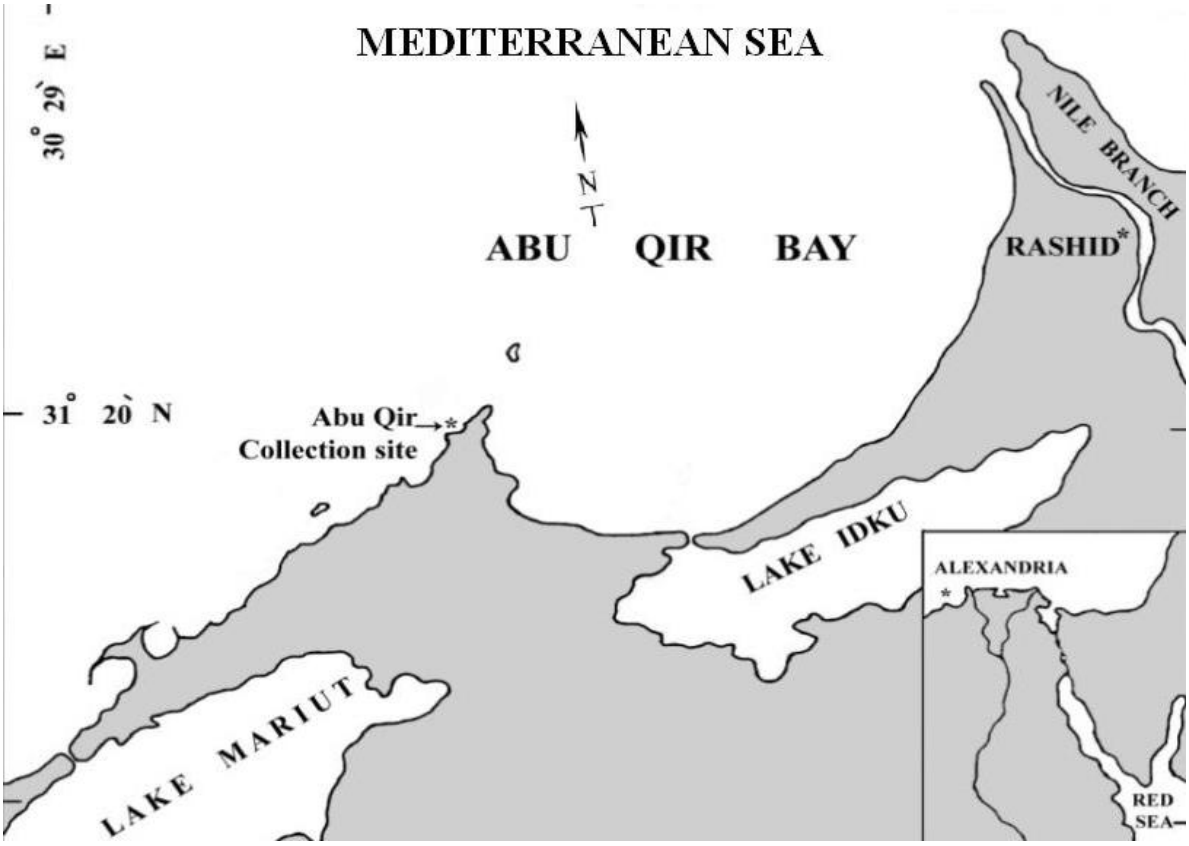


Figure 1. Map of Abu Qir showing collection site.

turally diverse antimicrobial metabolites from marine plants (Blunt et al., 2003, 2004, 2005; Faulkner, 2002). While, marine plant's metabolites have been studied extensively for their biomedical potential, their activities against human pathogens provide little information about their ecological role in antimicrobial activities and chemical structure of these antimicrobial compounds.

Alexandria has an extensive coast where, seaweeds from virtually all groups are present. The aims of this work were the search of novel compounds of potential antimic-robial value extracted from seaweeds, the study of the effect of seasonal variation on antimicrobial production, purification and elucidation of the structure of the antimicrobial compounds.

MATERIALS AND METHODS

Collection of algae

Three species of seaweeds from different divisions (*Jania rubens,* from Rhodophyta, *Ulva fasciata* from Chlorophyta and *Sargassum vulgare* from Phaeophyceae) were collected seasonally by hand in 2007 to 2008 from Rocky Bay of Abu Qir (N 31°19` E030°03`) (Figure 1). All samples were brought to the laboratory in plastic bags containing sea water to prevent evaporation. The algae were cleaned from epiphytes and rock debris and were given a quick fresh water rinse to remove surface salts. After collection, the samples were cleaned, air dried in the shade at room temperature (25 to 30°C) in the dark on absorbent paper and grounded to fine powder in an electrical coffee mill. The specimen from the collected seaweeds was preserved for identification and all the seaweeds were identified following Abbott and Hollenberg (1976) and Taylor (1985) and Aleem (1993).

Tested micro-organisms

Seven bacterial strains were obtained from the Culture Collection of Botany Department, Faculty of Science, Tanta University. They included *Bacillus subtilis, Staphylococcus aureus* and *Streptococcus aureus* as gram-positive bacteria, *Escherichia coli, Salmonella typhi* and *Klebsiella pneumoniae* as gram-negative bacteria and one yeast strain (*Candida albicans*) as yeast.

Preparation of the extracts

The extraction was carried out with70% acetone. The extraction was carried out by soaking the dried materials in 70% acetone (1:15 v/v) on a rotary shaker at 150 rpm at room temperature (25 to 30°C) for 72 h. The extracts from three consecutive soakings were pooled and filtered using filter paper (Whatman no. 4). The obtained filtrate was freed from solvent by evaporation under reduced pressure. The residues (crude extracts) obtained were resuspended in 70% acetone to a final concentration of 100 mg/ml and then stored at -20°C in airtight bottle.

Antimicrobial activity test

15 ml of the sterilized media (nutrient agar (Oxoid) for bacteria and Sabouraud dextrose agar for yeast) were poured into sterile caped

test tubes. Test tubes were allowed to cool to 50°C in a water bath and 0.5 ml of uniform mixture of inocula (10^8 CFU for bacteria and yeast) were added. The tubes were mixed using a vortex mixer vibrating at 1500 to 2000 rounds min^{-1} for 15 to 30 s. Each test tube's contents were poured onto a sterile 100 mm diameter Petri dish for solidification (Mtolera and Semesi, 1996).

The antimicrobial activity was evaluated using well-cut diffusion technique (El-Masry et al., 2000*).* Wells were punched out using a sterile 0.7 cm cork borer in nutrient agar plates inoculated with the test microorganisms. About 50 µl of the different algal extracts were transferred into each well. For each microorganism, controls were maintained where pure solvent was used instead of the extract. All the plates were incubated at 4°C for 2 h to slow the growth of microorganisms and give suitable time for the antimicrobial agent to diffuse. To prevent drying, the plates were covered with sterile plastic bags and incubated at 37°C for 24 h. (Mtolera and Semesi, 1996). The result was obtained by measuring the diameter of the inhibition zone for each well, and expressed as millimeter.

Statistical analysis

The results are presented as mean ± standard deviation of the mean (n = 3). The statistical analyses were carried out using SAS program (1989 to 1996) version 6.12. Data obtained were analyzed statistically to determine the degree of significance between treatments using one and three way analysis of variance (ANOVA) at P ≤ 0.01 and P ≤ 0. 001 levels of significance.

Column chromatography

Selected active crude extracts (2 g) were fractionated by column chromatography on silica gel (EDWC, 60-120 mesh). Column (2 cm × 40 cm) was set up in benzene with silica gel (30 to 40 g) and eluted with gradients of solvents from 10:1% of benzene: acetone to 1:10% benzene: acetone (Solomon and Santhi, 2008). The collected fractions were evaporated to dryness with a rotary evaporator and then, the dried samples were dissolved in pure acetone and assayed for their antimicrobial activity. The maximum absorption of the active fractions was measured by spectrophotometer (UV 2101/ pc) using quartz cuvette containing the different fractions. Different active fractions with same absorption maximum were pooled together (Solomon and Santhi, 2008). The active fractions were tested for purity using TLC (thin layer chromatography). The purified fraction was lyophilized and subjected to the following analyses in order to reveal its structure as far as possible:

UV-spectra

The UV-spectra of the tested material were determined using UV2101/pc spectrophotometer. The wavelength ranged from 200 to 800 nm.

The infrared spectra (IR)

Using Perkin-Elmer 1430 infrared spectrophotometer, the molecular structure of the antimicrobial material was partially identified. Since the antimicrobial material is liquid at room temperature, so it can be examined directly as a thin film, "neat", between two clean and transparent NaCl plates. The measurements were carried out at infra red spectra between 400 to 4000 nm.

Nuclear magnetic resonance (H^1NMR) spectra

The sample was dissolved in Dimethyl-d^6 sulfoxide (d^6 DMSO). The different functional groups were identified using NMR (JNMPMX60SI).

Mass spectra (MS)

A mass spectrophotometer (MS-5988) was used. The product was subjected to a steam of high energy of electrons at elevated temperature up to 100°C. The cleavage fragments were yielded which were characterized by mass/charge from mass spectra data.

RESULTS

J. rubens and *U. fasciat*a were present in all seasons, whereas, *S. vulgare* was present in spring and summer only. This indicated that a certain level of temperature is required for these species to grow in a massive quantity to facilitate the collection procedures.

Concerning the antimicrobial activities of the different seaweeds collected in the various seasons, the results in Table 1 showed that *U. fasciata* showed stronger activity than *J. rubens* in autumn. *S. aureus* was the most sensitive microorganism to *U. fasciata* extract. However, *K. pneumoniae* was the most sensitive microorganism to *J. rubens* extract.

In winter, the extract of *U. fasciata* also was more active than *J. rubens*, where *K. pneumoniae* was the most sensitive tested microorganisms for *U. fasciata* and *J. rubens* extracts. In spring, the extract of *J. rubens* was more active than *U. fasciata* and *S. vulgare*. With regard to *J. rubens,* it showed high antimicrobial activity against *B. subtilis* whereas, *K. pneumoniae* and *S. aureus* were the most sensitive to *U. fasciata* and *S. vulgare* extracts, respectively.

In summer, the extract of *U. fasciata* exhibited stronger antimicrobial activity than *J. rubens* and *S. vulgare,* respectively. The results showed that the extracts of *U. fasciata* inhibited all the tested microorganisms and *S. aureus* was the most sensitive microorganism to *U. fasciata* and *J. rubens* extracts. However, *B. subtilis* and *K. pneumoniae* exhibited higher activity for *S. vulgare*.

The obtained results show that the highest activity of the different seaweeds extracts were those collected in spring followed by winter, summer and autumn, respectively (Figure 2). The antimicrobial activity of the different species with respect to the different seasons could be arranged in the following order, *U. fasciata* in winter > spring > autumn > summer followed by *J. rubens* in spring > winter > autumn > summer and *S. vulgare* in spring > summer. The stated results indicated that the promising seaweeds for the production of the antimicrobial material was *U. fasciata* (Chlorophyta) that was collected in winter season against all the tested microorganisms. Therefore, we selected this species for further investigation.

The statistical analysis using three-way ANOVA confirmed that the variation in antimicrobial activities in rela-

Table 1. The antimicrobial activity of 70% acetone extract of seaweeds from different seasons against different tested microorganisms, measured as diameter of inhibition zone (mm).

Season	Microorganism	*B. subtilis*	*S. aureus*	*S. aureus*	*E. coli*	*S. typhi*	*K. pneumoniae*	*C. albicans*
	Seaweed	Diameter of inhibition zone (mm)						
Autumn	*J. rubens*	13±1	9±0	14.5±0.5	9.5±0.5	9.5±0.5	15.2±1.2	10±0
	U. fasciata	11±0	11±1	15±0	12±1	12±0.5	13.5±0.5	12±0
	S. vulgare	N.P.	N.P	N.P	N.P	N.P	N.P	N.P
Winter	*J. rubens*	14±0.1	12.1±0.1	13±0	16±0	12.2±0.7	17±0	12.7±0.2
	U. fasciata	22.2±0.2	19.3±1.2	20±1	19.5±0.5	21.8±1.0	24.6±0.5	19.5±0.7
	S. vulgare	N.P.	N.P	N.P	N.P	N.P	N.P	N.P
Spring	*J. rubens*	18±1	12.7±0.7	14±0	14.3±0.5	15±1	15.2±1.2	14.7±0.2
	U. fasciata	15.7±0.2	12.8±2.0	14.3±1.5	12.2±0.2	12.6±0.5	17.25±2.25	16.5±0.5
	S. vulgare	12±1	15.6±0.3	11.5±0.5	12±1	11±0	11.5±0.5	11.8±0.3
Summer	*J. rubens*	9.1±1.0	10.5±0.5	12.5±0	9±1	10±0	8.5 ± 0.5	8.2±0.2
	U. fasciata	10.6±0.5	12±1	14.5±1.5	11±1	14±1	12 ± 0.2	12±0
	S. vulgare	8.3±0.7	0±0	8±0	7.5±0	7.5±0.5	8.2 ± 0.7	0±0

N.P. = Not present; (±) standard deviation of the mean (n=3).

tion to seasons, seaweeds and microorganisms, and their interaction on antimicrobial activity was significant at $P \leq 0.001$ for all the treatments.

Purification and characterization of antimicrobial crude extract

Column chromatography

The results showed that 27 fractions were collected and only first four fractions had antimicrobial activity (Table 2). The UV absorption spectrums of these fractions were determined using spectrophotometer (UV 2101/ pc) at range of 200 to 800 nm. The obtained results are shown in Figure 3. The results indicated that the four fractions had the same absorptions peaks (three absorptions peaks at 333, 405 and 664 nm). Therefore, they pooled together. Thereafter, the different fractions were exposed to purity test using TLC technique (thin layer chromatography).

Thin layer chromatography (TLC)

The result showed that the examined four active fractions had approximately the same R_f value (Retention factor) and the statistical analysis using one-way ANOVA confirmed that the difference of R_f value of the four fractions compared with all the fractions sample was non-significant at $P \geq 0.01$ (Table 3). Therefore, from UV analysis and TLC technique, the four active fractions were pooled together.

Elucidation of the chemical structure of the purified material isolated from 70% acetone of winter collected *U. fasciata* extract

UV spectra of the antimicrobial material

Before measuring the UV spectrum, the different pigments and impurities were removed by filtration using charcoal. Then, the UV spectrum of the purified antimicrobial material isolated from *U. fasciata* was carried out in pure acetone. This spectrum showed one absorption peak at 333 nm, indicating the presence of an aromatic compound (Figure 4).

The obtained compound was examined for antimicrobial activity. The results showed that the compound still had antimicrobial activity indicating that the compounds which had peak at 405 and

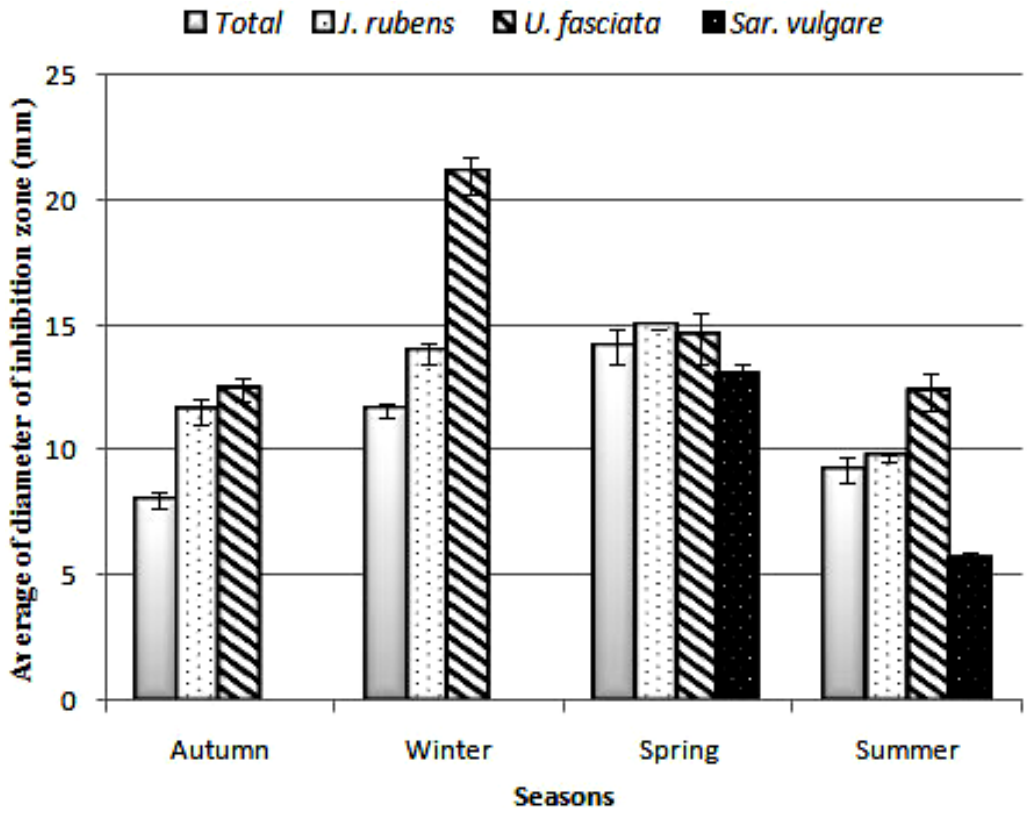


Figure 2. Antimicrobial activity of the different collected seaweeds that were collected in the different seasons.

Table 2. The antimicrobial activities of the different fractions obtained from the silica gel column chromatography against *K. pneumoniae.*

Number of fraction	Diameter of inhibition zone (mm)	Number of fractions	Diameter of inhibition zone (mm)
Fr.1	10 ± 0.023	Fr.15	-
Fr.2	13 ± 0.052	Fr.16	-
Fr.3	15 ± 0.034	Fr.17	-
Fr.4	17 ± 0.029	Fr.18	-
Fr.5	-	Fr.19	-
Fr.6	-	Fr.20	-
Fr.7	-	Fr.21	-
Fr.8	-	Fr.22	-
Fr.9	-	Fr.23	-
Fr.10	-	Fr.24	-
Fr.11	-	Fr.25	-
Fr.12	-	Fr.26	-
Fr.13	-	Fr.27	-
Fr.14	-		

(±)Standard error of the mean (n=3).

664 nm had not any antimicrobial activity. Therefore, we completed our investigation on the purified compound which had peak at 333 nm.

The infrared spectra (IR) of the antimicrobial material

The spectrum was subdivided into different regions, namely,

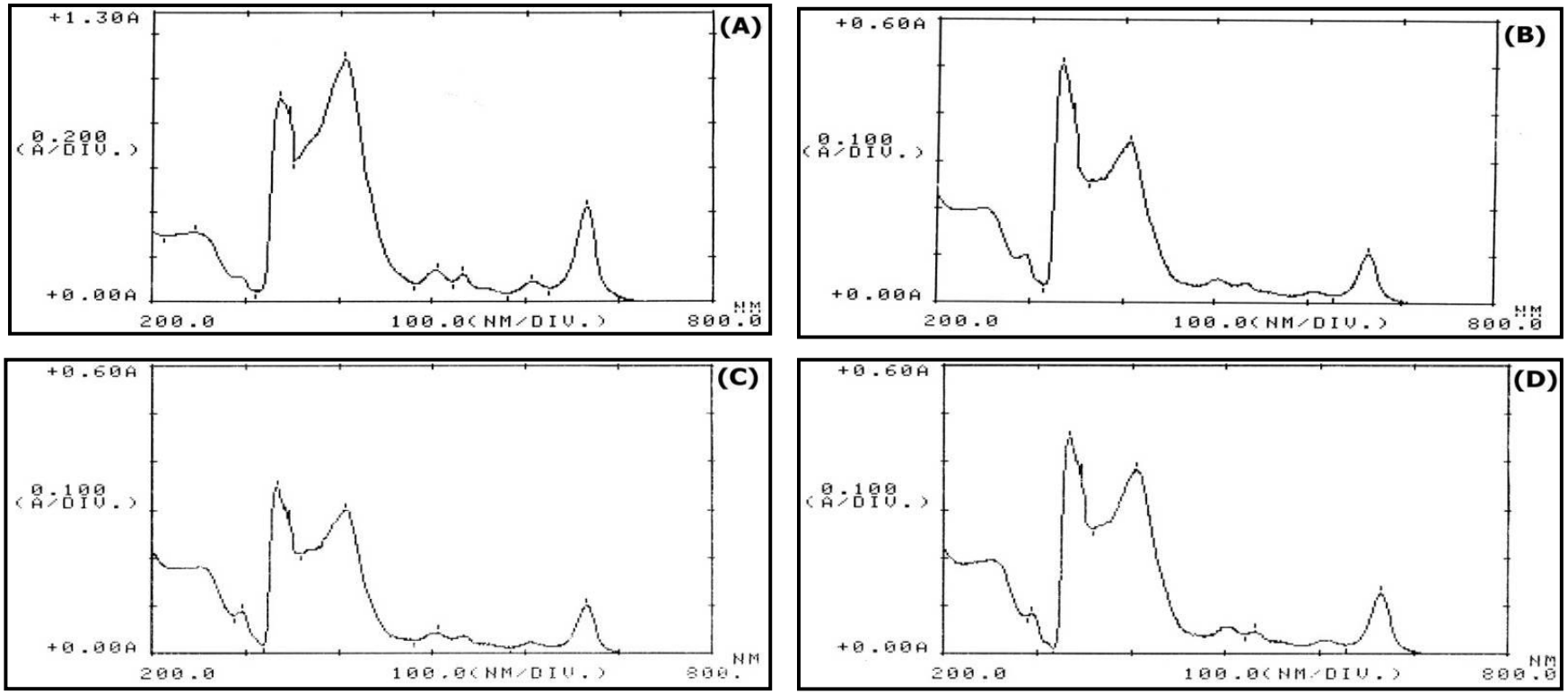


Figure 3. UV spectrophotometer scanning for the different active fractions separated by column chromatography.

Table 3. The retention factor of the spots from different fractions.

Sample	R_f
All fraction	0.38
Fraction1	$0.377^{(n.s.)}$
Fraction 2	$0.4^{(n.s.)}$
Fraction 3	$0.372^{(n.s.)}$
Fraction 4	$0.39^{(n.s.)}$
F-value	$5.67^{(n.s.)}$

$^{(ns)}$ Non significant at $P \geq 0.01$ using one way analysis of variance (ANOVA).

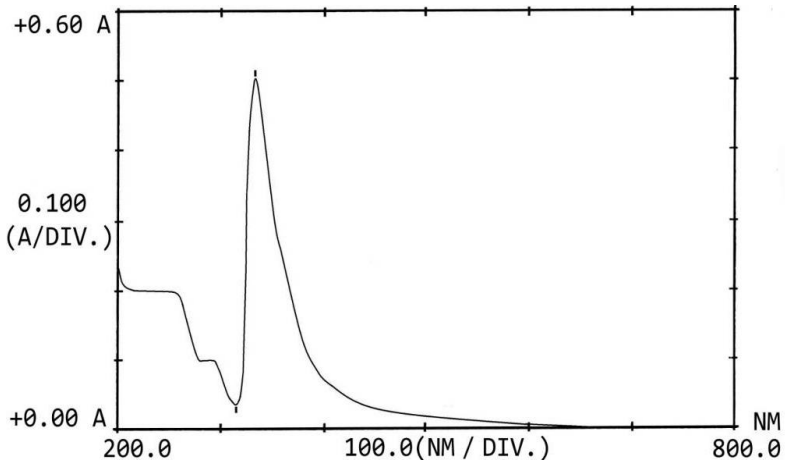


Figure 4. UV spectrum of the antimicrobial material produced by winter collected *U. fasciata* after purification by charcoal.

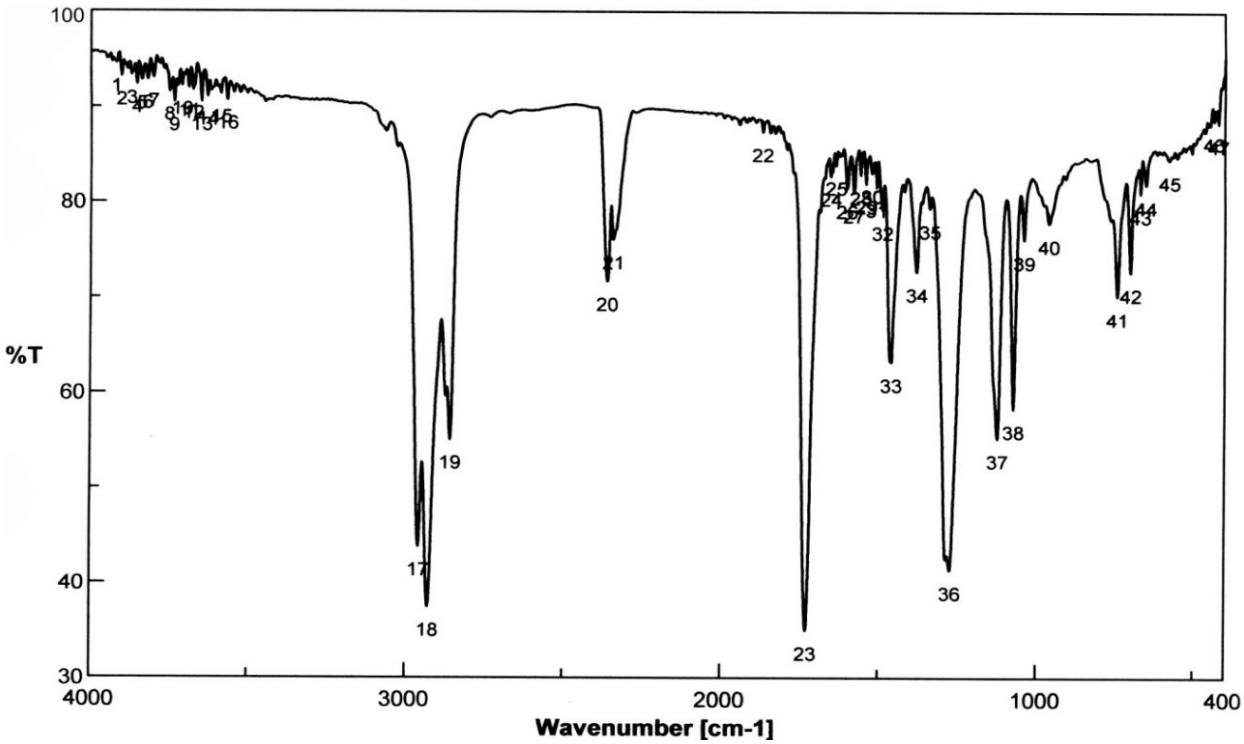


Figure 5. IR spectra of the purified antimicrobial material produced by winter *collected U. fasciata*.

the 2850 to 3050, 1710 to 1780, 1350 to 1470, 1020 to 1390 and 675 to 870 regions. The representative curve is shown in Figure 5.

Absorption in the 2850 to 3050 region: In this region, the υ CH aliphatic and υ CH aromatic strong stretch bands may appear. The IR revealed a strong band at 2958, 2927 and 2857 cm^{-1}, that can be attributed to the stretching vibrations of υ CH aliphatic and at 3000 to 3050 for υ CH aromatic group.

Absorption in the1710 to 1780 region: This region comprised one band due to the stretching vibration of the υ C=O of COOR group at 1729 cm^{-1}.

Absorption in the 1020 to 1390 region: This region comprised two bands due to the stretching vibration of the υ C-N at 1122 and 1072 cm^{-1} and in addition, one bands

bands due to the stretching vibration of the υ N-O of nitro at 1272.79 cm^{-1}.

Absorption in the 870 to 675 region: This region comprised 3 bands due to the stretching vibration of the υ phenyl ring substitution band at 742,700 and 6698 cm^{-1}.

Proton magnetic resonance spectra

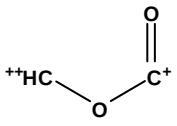
The ^1H NMR spectrum of the compound investigated was measured using in dimethyl-d^6 sulfoxide (d^6 DMSO) as solvent. The characteristic signals in ^1H NMR spectrum are represented graphically in Figure 6.

The signals were at δ 7.68 ppm (s,2H,2NH), at δ 6.89 to 7.47 ppm (m,3H of three aromatic protons), δ 5.320 ppm (s, 2H, CH=CH), δ 3.998 and 4.126 ppm (d, 2H, CH-NO_2), δ 3.342 ppm (s, 4H, 2 O-CH_2), δ 1.26, 1.6, 2.5, 2.73 ppm(s, 28H, 14 CH2) and at δ 1.9 and 2.1 (9H,3-CH_3).

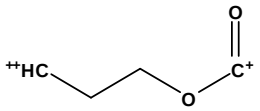
Mass spectra of the antimicrobial material

The mass spectrum fragmentation pattern of the compound investigated is shown in Figure 7. It reveals the presence of peak at m/z 662 of relative abundance characteristic of the parent compound. According to the earlier mentioned chemical analysis, the chemical structure of the purified antimicrobial material isolated from *U. fasciata* was:

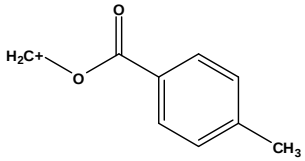
m/z: 57.03



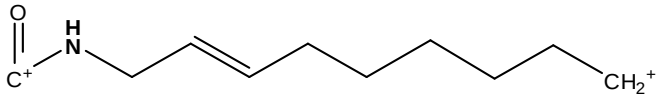
m/z: 85.03



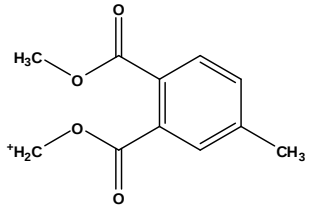
m/z: 149.17



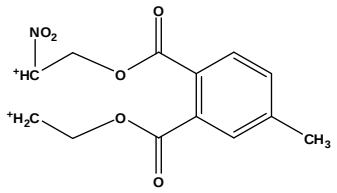
m/z: 167.25



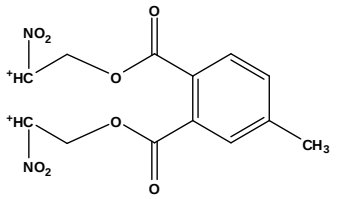
m/z: 207.20



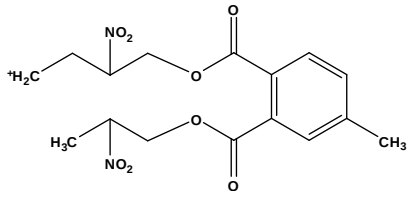
m/z: 279.24



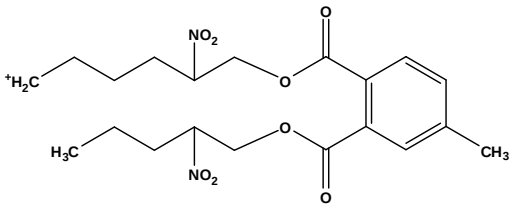
m/z: 324.24



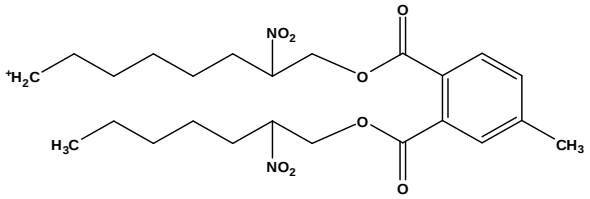
m/z: 367.33
m/z: 423.44



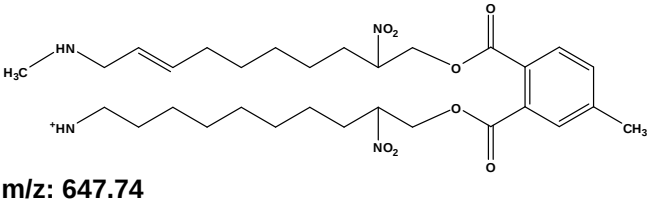
m/z: 479.54



m/z: 591.72



m/z: 647.74



According to the stated data, the suggested structure of the isolated antimicrobial substance should be as the following:

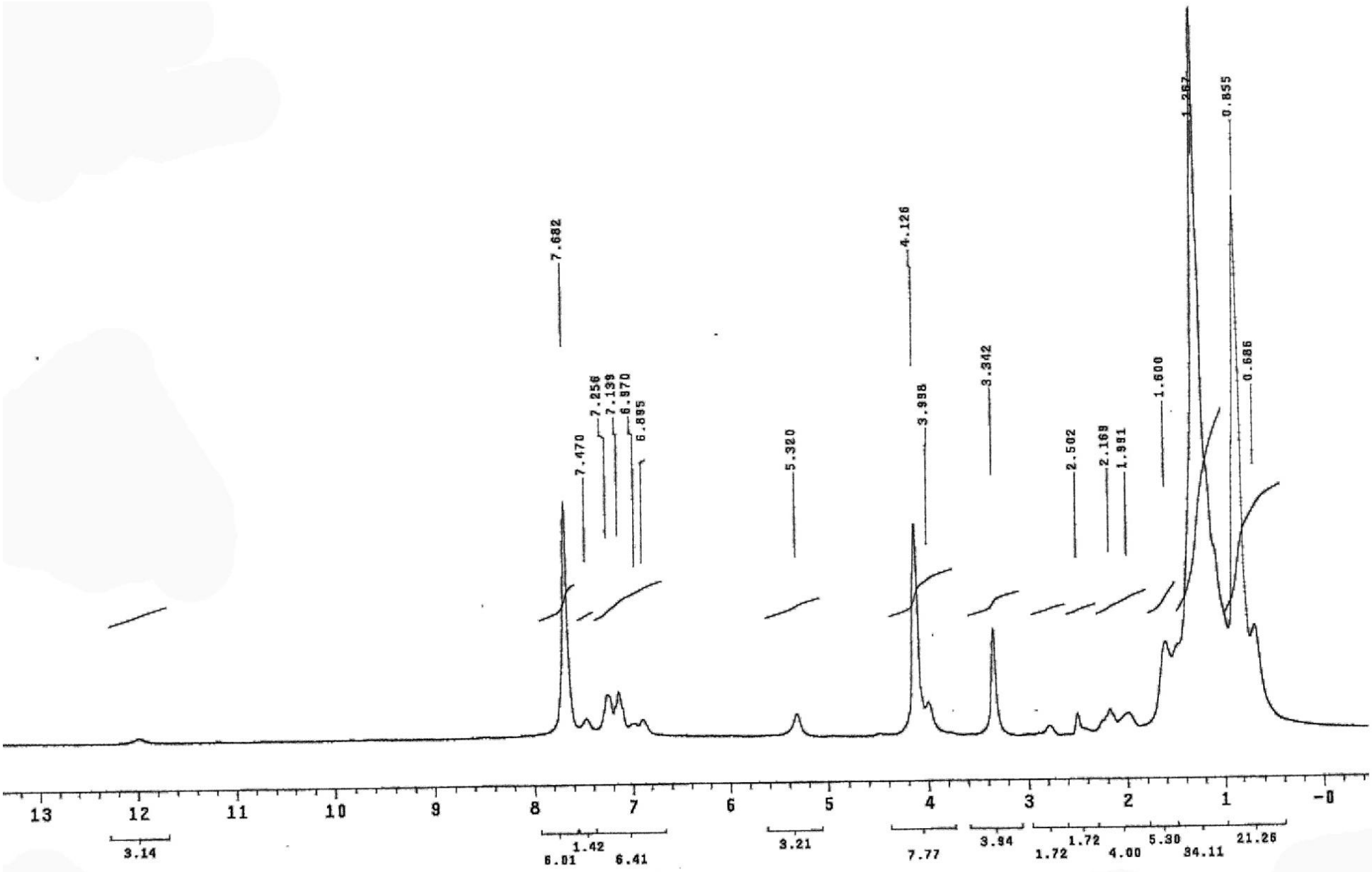


Figure 6. Proton magnetic resonance of the antimicrobial material produced by winter collected *U. fasciata*.

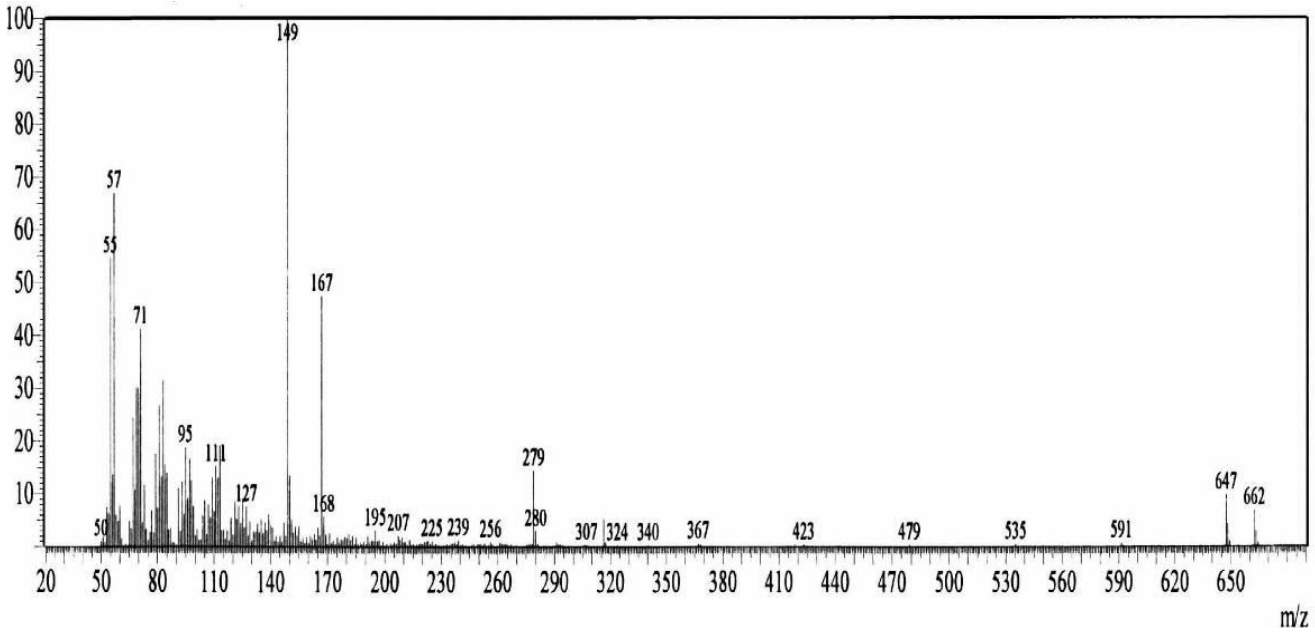
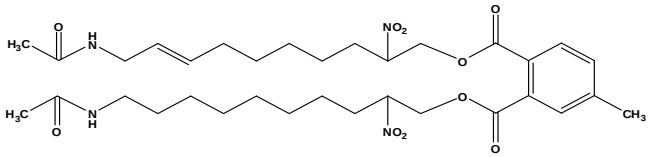


Figure 7. Mass spectra of the antimicrobial material produced by *U. fasciata*.



(*E*)-1-(10-acetamido-2-nitrodec-9-enyl), 2-(10-acetamido-2-nitrodecyl) 4- methyl phthalate. Chemical formula was $C_{33}H_{50}N_4O_{10}$; exact mass was 662.35; molecular weight was 662.77; elemental analysis was C: 59.80, H: 7.60, N: 8.45 and O: 24.14. This was the suggested chemical structure of the purified antimicrobial material isolated from *U. fasciata.*

DISCUSSION

This study is an endeavor for the study of the production, purification and structure elucidation of the antimicrobial compounds isolated from some species of seaweeds. Three species of seaweeds were tested for their capacity to produce antimicrobial substance. These species were collected from Abu-Qir, Alexandria coast, Egypt.

Lipid-soluble extracts from marine macroalgae have been investigated as a source of substances with pharmacological properties. Moreover, several different organic solvents have been used to screen algae for antibacterial activity (Mahasneh et al., 1995; Sukatar et al., 2006).

In this study, 70% acetone was used for extracting the bioactive compounds from different tested seaweeds and gave antimicrobial activity against all selected pathogens. This results are in accordance with those obtained by Wefky and Ghobrial (2008) and Fareed and Khairy (2008). It is well know that some species of macro-algae possess antibacterial activities against pathogenic bacteria (Kumar and Rengasamy, 2000; Selvin and Lipton, 2004; Tüney et al., 2006; Karabay-Yavasoglu et al., 2007; Salvador et al., 2007; Chiheb et al., 2009). The results reported by the earlier mentioned authors are in accordance with our data, which demonstrated that all the tested seaweeds had antimicrobial activity against the tested microorganisms. However, Salvador et al. (2007) and Gonzalez del Val (2001) detected that some seaweeds have not any antimicrobial activity in all the seasons. These differences in activity may be due to different developmental stages, locality, extraction methods, etc. Also, the antimicrobial activity depends on both algal species and efficiency on extraction of their active(s) principle(s)

In relation to the taxonomic groups, Reichelt and Borowitzka (1984) and Salvador et al. (2007) screened many species of algae for their antibacterial activity. They reported that the members of the red algae exhibited high antibacterial activity. In contrast, in our study, the green alga (*U. fasciata*) was the most active one. These results are in agreement with the results of Kandhasamy and Arunachalam (2008) who reported that green algae (Chlorophyta) were the most active taxa than others and Fareed and Khairy (2008) which showed that *U. lactua* (Chlorophyta) was more active when compared with *J. rubens* (Rhodophyta).

Some pure compounds isolated from seaweeds have been identified as natural antimicrobial compounds.

However, the relationship between their ecologic role and antimicrobial activities is not fully understood in many studies. Chemical defenses can be very specific or very broad, depending on the method of extraction, the orga-

nisms, season of algal collection, different growth stages of the plant, experimental methods, etc.

In our study, we focused on the possibility that the antimicrobial activity of the tested seaweeds will also fluctuate seasonally. As regards to seasonal variation of bioactivity, for all of the tested seaweeds, spring was the season with the highest activity against the test microorganisms followed by winter; these results are in accordance with those obtained from Atlantic samples by Hornsey and Hide (1974), from Mediterranean samples by Khaleafa et al. (1975) and Stirk and Reinecke (2007) who reported that seasonal variation in antibacterial activity was observed with the extracts which have antibacterial activity in late winter and early spring.

In this study, *U. fasciata* was the most effective seaweed species, having antibacterial activity throughout the year compared with other seaweeds screened for their antibacterial activity. *U. fasciata* inhibited the growth of all the tested microorganisms and this result is in agreement with Parekh (1978), who reported that the extract of *U. fasciata* was found to be inhibiting for both gram positive and gram negative bacteria. Also, Selvin and Lipton (2004) reported that the green alga *U. fasciata* exhibited broad-spectrum antibacterial activity.

The results showed that *U. fasciata* extracts of winter collection exhibited stronger antimicrobial effects followed by spring season than the other seasons and this agreed with Stirk and Reinecke (2007), who demonstrated that *U. fasciata* collected in winter and spring seasons were active against the tested organisms compared with other seasons. This may be influenced by the seasonal variation as extracts of *U. fasciata* from winter and spring collection were more potent as compared with summer and autumn collections. The former represents the peak growing and reproductive season, while the later is the stasis and senescence period of *U. fasciata* growth. The highest antimicrobial action of winter collection is possibly due to the elevated biochemical constituents during the growing and reproductive phase of the *U. fasciata*. This hypothesis is further strengthened by some worker (Hornsey and Hide, 1974; Daly and Prince, 1981; Moreau et al., 1984; Rao and Indusekhar, 1989; Muñoz, 1992).

In this study, the antimicrobial material extracted from *U. fasciata*, which was collected from column chromatography was purified with charcoal and subjected to UV analysis. It showed maximum absorption spectra at 333 nm. Accordingly, the composition of the active antimicrobial material was suggested to contain aromatic ring.

The infrared (IR) spectroscopy indicated the presence of many functional groups in the antimicrobial material which were υ CH aliphatic at 2958, 2927 and 2857 cm^{-1}, υ CH aromatic at 3000 to 3050, υ C=O of COOR group at1729cm^{-1}, υ C-N at 1122 and1072 cm^{-1}, υ N-O of nitro at 1272 cm^{-1} and υ phenyl ring substitution band at 742.46, 700.033 and 669.178 cm^{-1}.

The ^{1}H NMR spectrum showed two protons of two (NH), three protons of aromatic protons, two protons of (CH=CH), two protons of two (CH-NO2), two protons of two (O-CH2), 28 protons of 14 (CH2-) and nine protons of three (-CH3). The mass spectroscopy of our antimicrobial material indicated that the molecular weight was 662.

According to the obtained data, the compound is an aromatic compound having the following structure $C_{33}H_{50}N_4O_{10}$ [(E)-1-(10-acetamido-2-nitrodec-9-enyl) 2-(10-acetamido-2-nitrodecyl) 4-methylphthalate]. This is the first evidence for the isolation of phthalate esters derivative from green seaweeds (*U. fasciata*) and that has broad antimicrobial activity. However, some worker isolated it from brown algae. Cho et al. (2005) isolated di-*n*-octylphthalate which has antifouling activities from the brown seaweed *Ishige okamurae*. Also, Ganti et al. (2006) isolated phthalic acid from *Sargassum confusum*.

These previous reports led us to suggest that the origin of the phthalate esters was natural and can be synthesized by living organisms not derived from artificial products or a contaminant from environment.

Finally, we conclude that macroalgae from Abu Qir coast in Alexandria could be considered as potential sources of bioactive compounds and the production of these compounds are affected by seasons. Our study indicated that the activities of different seaweeds for the production of antimicrobial substances in various seasons could be arranged in the following seasons spring > winter > summer >autumn and we obtained the highest activity from the winter collected *U. fasciata*. Thus, the production of the antimicrobial substance by seaweeds is season and species dependant. The structure of this antimicrobial material is $C_{33}H_{50}N_4O_{10}$ [(E)-1-(10-acetamido-2-nitrodec-9-enyl) 2-(10-acetamido-2-nitrodecyl) 4-methylphthalate], which is a strong anti-microbial activity against the tested human pathogenic microorganisms.

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Moroccan rock phosphate solubilization during a thermo-anaerobic grassland waste biodegradation process

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In order to investigate the presence of thermo-tolerant rock phosphate (RP) solubilizing anaerobic microbes during the fermentation process, we used grassland as sole organic substrate to evaluate the RP solubilization process under anaerobic thermophilic conditions. The result shows a significant decrease of pH from 6.5 to 4.8, and solubilizing from 7 to 15.8% of the phosphorus from the RP in the reactors after 90 days of incubation at 45°C. In these conditions, the organic acids produced were qualitatively and quantitatively identified as: acetic, butyric and propionic acids. This biological RP solubilization is due to the presence of a single thermo-tolerant bacterium isolated and identified as *Bacillus subtilis* from the anaerobic reactors. This *B. subtilis* strain was shown to be able to solubilize RP in liquid cultures containing insoluble RP as sole phosphate source. The mechanisms involved in these weathering processes confirmed the production of organic acids which were identified and quantified. This study is expected to lead to the development of novel, non-polluting farming practices by entering in the formulation of novel multi-functional biofertilizer by inoculating this thermo-tolerant phosphate-solubilizing bacterium into agricultural wastes as a practical and environmental strategy.

Key words: Grassland, phosphate, solubilization, *Bacillus subtilis*, thermo-anaerobic conditions.

INTRODUCTION

Phosphorus (P) is an essential element for life. After nitrogen, P is the second major element effecting plant growth and yield. P contributes to the biomass construction of micronutrients, the metabolic process of energy transfer, signal transduction, macromolecular biosynthesis, photosynthesis, respiration chain reactions and physiological chemical process for plant as well as for seed maturation (Shenoy and Kalagudi, 2005). Unfortunately, the concentration of P in soil solution is very low; varying from 0.001 mg/l in very poor soils to 1 mg/l in heavily fertilized soils (Antoun, 2012).

Therefore, phosphatic fertilizers, such as costly chemical fertilizers that contain large amounts of soluble P, have been applied to the agricultural fields to maximize

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production (Shenoy and Kalagudi, 2005). However, the soluble P in phosphatic fertilizers which is easily and rapidly precipitated to insoluble forms may become unavailable to the plant because of mineral phase reprecipitation (Wakelin et al., 2004). It is estimated that because of P-fixation in soil, plants will take up the year of application, only 10 to 15% of the soluble P added as fertilizers or manure (Brady and Weil, 2008). The unmanaged use of phosphatic fertilizers has increased agricultural costs and instigated a variety of environmental problems (Del Campillo et al., 1999). Therefore, the concept of adding phosphate-solubilizing microorganisms (PSMs) to rock phosphate (RP), a finite non-renewable resource and natural fertilizers, as providers of soluble P presents an economically and environmentally promising strategy (Antoun, 2012; Chang and Yang, 2009).

PSMs may play a major role in developing a sustainable use of P resources and in biogeochemical P cycling in natural and agricultural ecosystems. PSMs can transform the insoluble P to soluble forms by acidification, chelation, exchange reactions, and polymeric substances formation (Delvasto et al., 2006).

Therefore, the use of PSMs in agricultural practice would not only offset the high cost of manufacturing phosphatic fertilizers but would also enhance the solubilization of reprecipitated soil P for crop improvement (Shekhar et al., 2000). Many genera and species of bacteria have been described as PSMs (Hamdali et al., 2008; Yu et al., 2012).

However, all PSMs studied and applied to date have been mesophiles that could only be used under mesophilic and aerobic conditions. These types of microorganisms are not appropriate for the preparation of multifunctional biofertilizer at the high temperatures that occur for decomposing complex organic wastes (Yang, 2003).

Since cellulose is mostly present in plant cell walls, which are very difficult to degrade, only a small fraction of all microorganisms that are specialized for plant cell wall degradation can hydrolyze cellulose (Li et al., 2009), probably because it is present in recalcitrant cell walls (Wilson, 2011). To date, a few study described that anaerobic cellulolytic thermophillic bacteria have a very effective plant cell wall degradation system (Blumer-Schuette et al., 2010).

In the present study, we investigated the presence of thermo-tolerant RP-solubilizing bacteria, during the fermentation of grassland wastes as the sole organic substrate. The effects of these bacteria on RP solubilization through the anaerobic process have also been determinate. The solubilization mechanism and the identification of the selected bacteria were achieved.

MATERIALS AND METHODS

The rock phosphate sample

Rock phosphate originating from the Youssoufia phosphate mine (RP^Y, insoluble rock phosphate powder), was ground with pestle and mortar and passed through a 100 µm sieve. Its chemical characteristics were as follows: 31.4 g 100/g P_2O_5, 50.2 g 100/ g CaO and 2 g 100/ g total organic carbon (Khaddor et al., 1997).

Substrate preparation and fermentation experiments

The grassland experiment was conducted in a greenhouse at 24°C under artificial light for 16 h and at 17°C for 8 h in darkness. The experiment was started on 12 June 2010 on a poorly drained Stagno-Gleyic Luvisol (FAO classification) with rather wet and cool weather at the beginning of the investigation. After two weeks, the sward was maintained at a height of about 5 cm. The pasture was dominated by perennial ryegrass (*Lolium perenne* L) and common bent (*Agrostis capillaries* L). The C and N contents of the grassland topsoil (0-0.15 m) were 1.6 and 0.17%, respectively, with soluble organic carbon (extracted with 0.05 M K_2SO_4) of 87.5%. The grassland was oven dried at 70°C and used as organic substrate for the fermentation experiments.

Dried substrate (165 g) was placed in 5 L Erlenmeyer flasks containing 2 g of RP^Y as sole phosphate source and completed to 4 L with sterile distilled water. Cultures were grown in triplicate on a laboratory digester maintained at 45°C for 90 days in completely filled flasks with rubber stoppers and with shaking at 100 rpm in an incubation shaker to minimize aggregation of the bacteria. Anaerobic conditions were achieved through the consumption of residual oxygen. Similar experiments were carried out with no phosphate source incubated under the same conditions. Samples of 20 ml of each culture were collected periodically (every 10 days). After then, the pH value of the medium was determined with a pH meter equipped with a glass electrode. The culture supernatant obtained by centrifugation (10,000 rpm, 15 min) was passed through a 0.45 mm Millipore filter. The inorganic phosphate content of culture filtrate and organic acids analysis were determined by high performance ion chromatography, HPIC, Type DIONEX Dx-120 (AS11-HC column, injection rate: 2.3ml / min, detector: conductimetric cell).

Isolation of rock phosphate solubilizing bacteria under anaerobic process

At different state of treatment, 0.1 ml of the liquid suspension was sampled and plated in triplicate on the surface of nutrient agar (Difco, USA) to isolate the bacteria. The pH was adjusted to 7 and the medium was sterilized at 121°C for 20 min. After plating under the anaerobic conditions in the presence of Methylen blue as indicator, the agar plates were incubated for 3 days at 45°C.

Selection of phosphate solubilizing bacteria was carried out by plating the nutrient agar isolate on the solid NBRIP medium (Nautiyal, 1999) containing (g/l) Glucose : 10; $MgCl_2.6H_2O$: 5 ; $MgSO_4.7H_2O$: 0.25 ; KCl : 0.2 ; $(NH_4)2SO_4$: 0.1; agar : 15; pH : 7.45; and RP^Y : 0.5 as sole phosphate source. After plating, the agar plates were incubated for three days at 45°C in anaerobic atmosphere. Spores of the phosphate solubilizing bacteria (PSB) isolate and able to show the most active growth on NBRIP were stored in 20% (w/v) sterile glycerol at -20°C.

Estimation of the ability of the selected PSB to release soluble phosphate from RP^Y

Three culture replicates were inoculated with 10^6 spores/ml of the PSB isolate and grown for nine days at 45°C on a rotary shaker (180 g/min) in 250 ml Erlenmeyer flasks containing 50 ml of liquid NBRIP medium with 0.5 g/l RP^Y and maintained under anaerobic conditions. Cultures were centrifuged at 10,000 *g* for 10 min and the pH of the supernatant was measured every day. The superna-

tant was analyzed for P_2O_5 content by the HPIC method as described above. Similar measures were carried out in non-inoculated flasks incubated under the same conditions.

Taxonomic study of the selected strain

Morphological and physiological characterization of selected isolate

The isolate was examined for several phenotypic characteristics. Unless otherwise stated, the tests were carried out in tryptic soy agar (TSA) medium at 32 and 45°C incubation temperature. Flagella were stained using the method of Rhodes. Spores were stained according to the Schaeffer-Fulton method with five days culture on TSA medium in aerobic and anaerobic conditions. Catalase and oxidase production, aerobic nitrate and nitrite reduction and acid-production profiles from carbohydrates were obtained with an API 50CH system (bioMérieux) after growth in 50 CHB/E medium, as described by Logan and Berkeley (1984). Antimicrobial susceptibility was tested in TSA medium according to the method of Bauer et al. (1966).

Amplification and sequencing of the 16S rDNA of the selected strain

The strain isolated in this work was grown on Luria-Bertani medium as standard cultures of *Bacillus sp.* The isolate was incubated in completely filled flasks with rubber stoppers and with shaking at 100 rpm. Inoculation was performed aerobically with an aerobically grown overnight culture with an optical density at 578 nm of 0.3. Anaerobic conditions were achieved after a short time through the consumption of residual oxygen by the inoculated bacteria. The cells for preparations of DNA were harvested after 3 h in the midst of the exponential growth phase.

The 16S rRNA gene was amplified by PCR using standard protocols (Saiki et al., 1988) and the forward primers 16F27 (5'-AGAGTTTGATCATGGCTCAG-3') and the reverse primer 16R1488 (5'-CGGTTACCTTGTTAGGACTTCACC-3') (both from Pharmacia). The PCR products were purified using the Microcon Qiaquick spin-gel extraction kit (Qiagen). Direct sequence determinations of PCR-amplified DNAs were made with an ABI PRISM dye-terminator, cycle sequencing ready-reaction kit (Perkin-Elmer) and an ABI PRISM 377 sequencer (Perkin-Elmer) according to the manufacturer's instructions. The sequence obtained was compared to reference 16S rRNA gene sequences available in the GenBank and EMBL databases obtained from the National Centre of Biotechnology Information database using the BLAST search. Phylogenetic analyses were made using MEGA version 2.1 (Kumar et al., 2004) after multiple alignment of the data by CLUSTAL V (Thompson et al., 1997). Distances and clustering were determined using the neighbour-joining and maximum-parsimony algorithms. The stability of the clusters was ascertained by performing a bootstrap analysis (1000 replications).

Statistical analysis

All experiments were carried out in triplicate or more. All data are reported as means ± SD (standard deviation). The Independent-Samples t-test was used to compare means and the variance homogeneity determination (ANOVA) was conducted with the General Linear Model using type II sum of squares and Tukey's Honestly Significant Difference (P = 0.05) using statistical analysis system software (SAS Institute, 2002).

RESULTS AND DISCUSSION

Isolation of bacteria able to use RP as sole phosphate source

After plating and incubation period of liquid samples at the final phase of the fermentation experiments, only one bacterium isolate could grow when plated on the solid nutrient medium. This thermo-tolerant anaerobic isolate could use RP when plated on the solid NBRIP medium containing RP^Y as unique phosphate source. This is unexpectedly low since the cultures were maintained during the fermentation anaerobic process and most of the phosphate in this biotope is in an insoluble form. Up to date, only a few works reported that some thermo-tolerant phosphate solubilizing microbes were isolated aerobically from compost plants and biofertilizers (Chang and Yang, 2009; Xiao et al., 2008).

Abilities of the selected isolate to release soluble phosphate from RP

Fermentation experiments

The soluble phosphorus content increased significantly between 0 and 10 days from 19.6 ± 0.1 to 36.7 ± 0.2 mg/l, then decreased slightly at 20 days and re-increased after, up to 43.3 ± 0.1 mg/l at 90 days (Figure 1A). The microbial activities were amplified more vigorously at early fermentation phase, resulting in the consumption of soluble phosphorus for microbial growth. These results indicate that thermo-tolerant PSMs can increase the soluble phosphorus content and contribute to the solubilization of RP during the fermentation process. The RP solubilization rate ranged from 7 to 15.8% and increased during the thermophilic anaerobic fermentation experiments (Figure 1A). It was reported that *Bacillus smithii* F18 presented the highest soluble phosphorus percentage (5.3 ± 0.6%) of the total phosphorus after 56 days of composting. In comparison, under aerobic conditions, the control (non-inoculated compost) had the lowest percentage of 2.9 ± 0.2% (Chang and Yang, 2009). Similarly, a moderately thermophilic bacterium *Acidithiobacillus caldus* achieved a phosphorus solubilizing rate of 27.6% in shake flasks containing elemental sulfur (S0) as an energy substrate and only 2.19% for the same system without the additional S0 (Xiao et al., 2011).

Growth in flasks in NBRIP liquid medium

The amount of P solubilized increased with time, and at the end of the incubation period (9 day), the values obtained were significantly different from those of the control (1.6 mg/l of soluble P), irrespective of P source

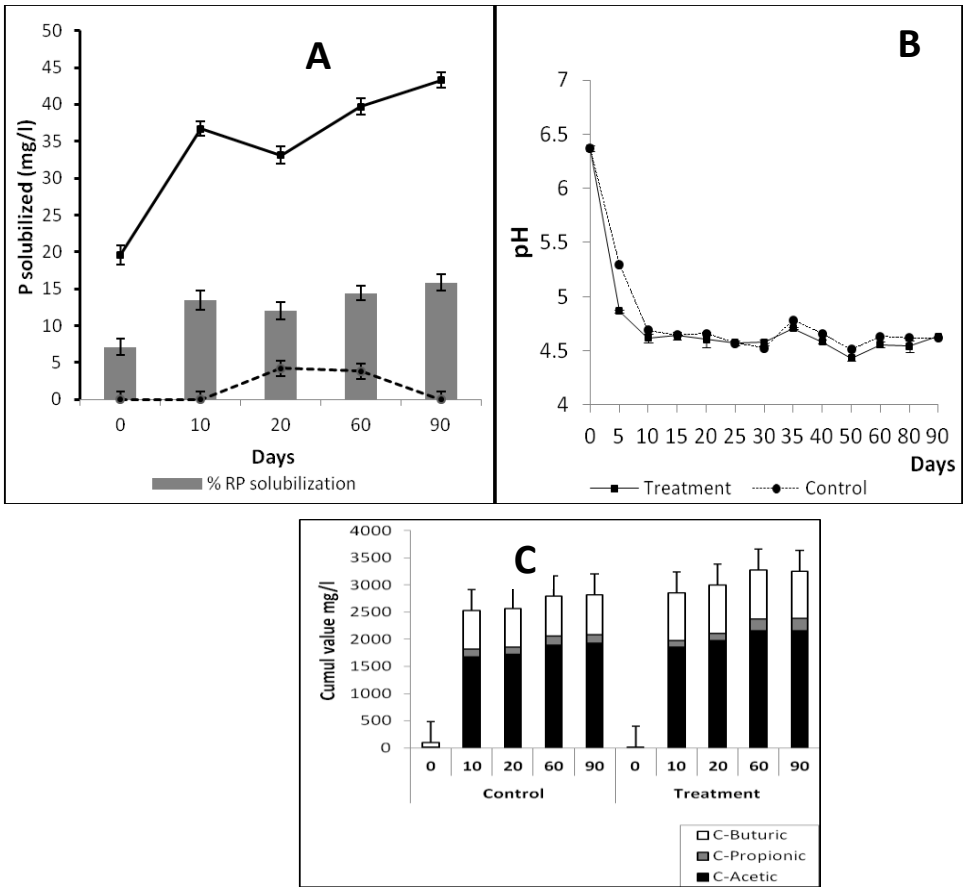

Figure 1. Fermentation experiments in bioreactor with Grassland containing 0.5 g/l RP^Y (Treatment) or without RP^Y (Control) incubated for 90 days under the same conditions. **(A)** Concentration of soluble phosphate released from rock phosphate in the supernatant of cultures. **(B)** Evolution of the pH in the supernatant of cultures. **(C)** Concentration of organic acids produced in the supernatant of cultures. Data points are means and vertical bars are standard deviations (n=3).

(Figure 2A). Phosphate release ranged from 2 to 13 mg/l in the growth medium NBRIP. This showed that the selected anaerobic thermo-tolerant isolate had effectively converted the inorganic insoluble RP into a soluble form. It was indicated that the application of the moderate thermophile of *A. caldus* was an effective method to solubilize phosphorus from RP than that obtained with the mesophile, *Acidithiobacillus thiooxidans* (Xiao et al., 2011). Chang and Yang (2009) reported that an aerobic thermo-tolerant *Bacillus smithii* F18 had the highest RP solubilizing activities with 544.2 ± 30.2 µg/ml of soluble phosphate in Pikovskaya's broth at 50°C.

RP solubilization mechanism by the selected isolate

Fermentation experiments

On Petri dishes, the isolates were surrounded by a clear halo, characterizing microorganisms producing organic acids on the NBRIP media (Nautiyal, 1999). Noticeable acidification of the growth medium was observed during the anaerobic digestion of grassland (Figure 1B) suggesting that the process of RP solubilization involved the excretion of organic acids.

Organic acid production is the main mechanism by which PSMs mobilizes P from sparingly soluble phosphates (Khan et al., 2007). Solubilization of phosphates may result from the drop in pH or from cations chelation by organic acids (Antoun, 2012). In these experiments, pH decreased significantly from 6.5 to 4.8 and remains substantially unchanged until the end of the treatment (90 days) (Figure 1B). Thus, cellulose is a polymer of glucose and it is readily hydrolyzed during acidogenesis (Huang et al., 1986). The pH value of 4.8 indicates microbiological activity and the presence of thermo-cellulolitic acidophilic bacteria. Xiao et al. (2011) showed that the phosphate solubilizing ability of *A. caldus* was the most effective when carried out under pH 2.5 at 45°C. A few studies have reported the presence of cellulolytic bacteria

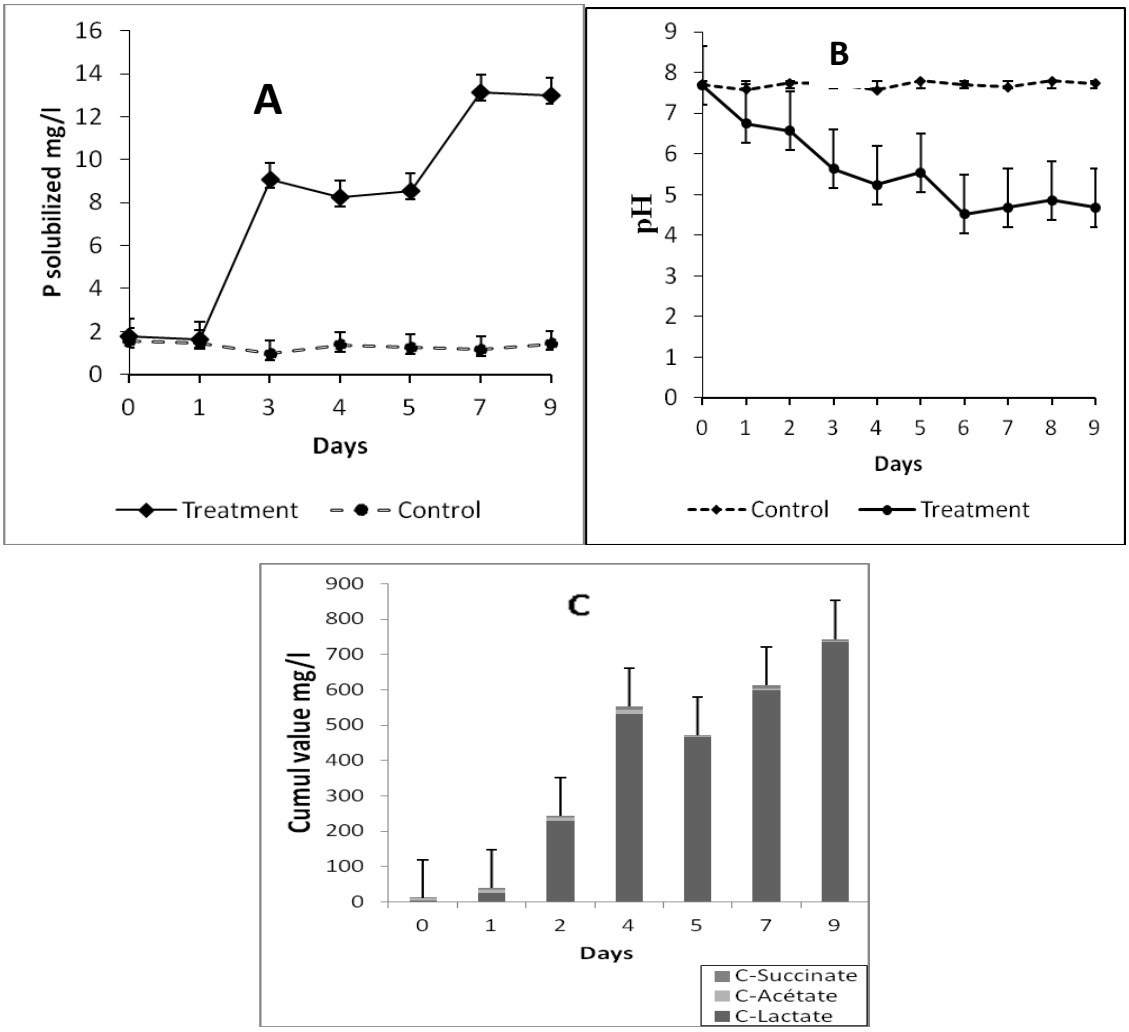

Figure 2. Culture of *Bacillus subtilis* in flasks in NBRIP liquid medium containing 0.5 g/l RP^Y (Treatment) or without RP^Y (Control) incubated for 9 days under the same conditions. **(A)** Concentration of soluble phosphate released from rock phosphate in the supernatant of cultures. **(B)** Evolution of the pH of the culture supernatant. **(C)** Concentration of organic acids produced in the supernatant of cultures. Error bars represent standard deviations of the mean values of the results of three independent culture replicates.

able to grow on native cellulose as only carbon source (Schwarz, 2001). In addition, cellulolysis was considered more effective and faster with thermophilic than mesophilic bacteria (Leschine and Canale-Parola, 1983). It was reported that *Acidothermus celluloticus* was a rare case of cellulolytic species; acidophilic and thermophilic able to grow at optimal pH of 5 and which could grow at pH 3 on cellulose substrate in the medium (Bergquist et al., 1999). In this study, the following acids (acetic, butyric, propionic acids) were found in the culture filtrates during the anaerobic fermentation process, using HPIC chromatography (Figure 1C). These identified organic acid presented after 60 days of anaerobic fermentation the concentration of 3300, 2400 and 2200 mg/l of butyric, propionic and acetic acid, respectively (Figure 1C). It was reported that the acidophilic bacteria can acidify the medium at pH values from 3 to 4 and can transforms sugars into lactic, acetic, propionic, byturic acids, alcohol and CO_2 (Kawagoshi et al., 2005). High concentrations of organic acids and therefore relatively low pH are usually encountered in anaerobic ecosystem environments (Goodwin and Zeikus, 1987).

Growth in flasks in NBRIP liquid medium

Compared with the solubilizing system for RP without bacterium, the pH of the culture with the bacterium was obviously lower during the solubilizing process in the NBRIP liquid medium (Figure 2B). There was a gradual decrease of pH from 7.7 up to 4.8 at day 9 as a result of the consumption of acid by the proton attack on RP. This

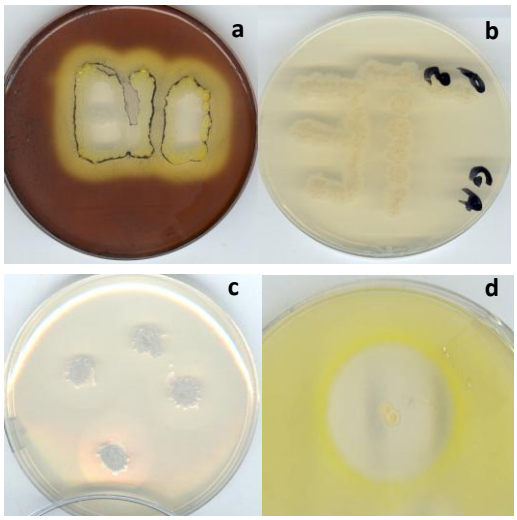

Figure 3. (a) Starch degradation showed by iodine -test of the selected isolate. **(b)** Cream pigmented colonies. **(c)** RP solubilization by the selected isolate on NBRIP medium by acid production mechanism. **(d)** Antibacterial activity against *Micrococcus luteus* ATCC 381.

result confirms the ability of the selected isolate to solubilize RP by producing organic acids. Research of organic acids in the liquid NBRIP culture revealed the presence of acetic, lactic and succinic acids at different concentration (Figure 2C). The presence of formic, propionic, butyric, pyruvic, glutaric, malic, tartric and oxalic acids was signaled at only trace concentration (< 2 mg/l). Lactic acid is the most produced from 2 mg/l to more than 700 mg/l after 9 days of incubation. It was reported that a strong correlation between pH and soluble P concentration, as well as total organic acid production and the P solubilized was observed with the PSB strain under aerobic conditions (Yu et al., 2012). Similarly, it was indicated that a thermophilic aerobic bacterium of *A. caldus* have the best ability to produce organic acids than the mesophilic bacteria (Xiao et al., 2011).

Taxonomic characterization of the selected isolate

Identification of isolated *Bacillus* may follow one of several methods of classification. In this study, the selected strain was tested for taxonomical identification using morphological, cultural, physiological and biochemical criteria as well as other features (Claus and Berkeley, 1986). Cells are rods Gram-positive, cream pigmented, anaero-aerobic, occurring singly or in pairs and occasionally in short chains or filaments. They are motile by peritrichous flagella. In aerobic condition at 30°C, the endospores are mainly ellipsoidal and lie in subterminal positions. Catalase and oxydase are positives. However, when grown on in anaerobic condition at 45°C, the endospores are absents. This result is consistent

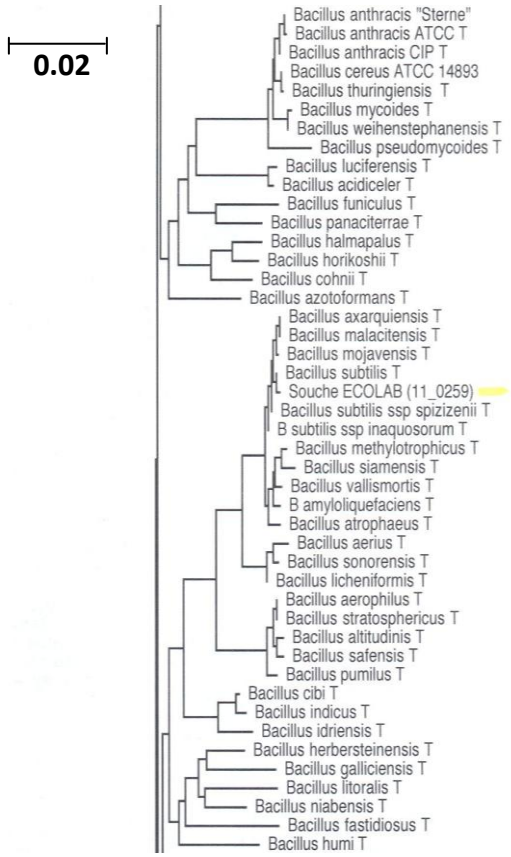

Figure 4. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of the selected isolate. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 1 nt substitutions per 100 nt.

with those of Espinosa et al. (2001).

The bacterium grows within a temperature range of 20 to 50°C and pH values comprise between 4.5 and 10. It is halo tolerant, being able to growth in salt concentrations from 0 to 7.5% w/v. The selected strain showed anti-fungal and antibacterial activities. It reduces nitrate aerobic and anaerobically. Starch and cellulose are used. Citrate is used as sole carbon and energy source. Several phenotypic features are shown in Figure 3. The selected isolate was colistin sensible and predicted to belong to the genus *Bacillus*.

The sequencing of the 16S RNA of this strain confirmed this classification. Indeed comparisons of the 16S rRNA gene sequences of the selected strain and those available in the GenBank database indicated that it is phylogenetically closely related to, respectively, *B. subtilis* (98% sequence homology), *Bacillus mojavensis* (97% sequence homology), *Bacillus malacitensis* (97% sequence homology) and *Bacillus axarquiensis* (96% sequence homology) (Figure 4). The phylogenetic tree, constructed using the neighbour-joining method is depicted in Figure 4.

Conclusion

Rock phosphate bio dissolution is due to the presence of a single thermo-tolerant strain of bacteria (*B. subtilis)* isolated by the NBRIP test from the reactors. Tests confirm that *B. subtilis* strain is able to grow anaerobically and can hydrolyse the cellulose substrate. To our knowledge, this study is the first report showing the ability of bacteria to solubilize RP and to degrade cellulosic substrates under thermophilic and anaerobic conditions. The mechanism involved in the RP solubilization is a consequence of proton release from organic acids in the aqueous phase of the fermentation digester.

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RAPD-PCR analysis of some species of *Euphorbia* grown in University of Baghdad Campus in Jadiriyah

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This study attempts to identify species of *Euphorbia* (*Euphorbia peplus*, *Euphorbia helioscopia*, *Euphorbia granulata* and *Euphorbia hirta*) grown in University of Baghdad Campus in Jadiriyah and determine the genetic polymorphism among them by using DNA markers generated by polymerase chain reaction (PCR). Total genomic DNA of species studied was extracted from dry seeds by using commercial kit. Molecular analysis was performed by using nine random markers in random amplified polymorphic DNA (RAPD-PCR) technique. RAPD-PCR analyses based on three primers A13, C05 and D20 gave results in term of amplification and polymorphisim for the four species studied. The genetic polymorphisms value of each primer was determined and ranged between 47 to 84%; primer A13 produced the highest percent of genetic polymorphism compared with primer C05. RAPD-PCR technique confirmed the isolation of the four species of *Euphorbia* obviously.

Key words: *Euphorbia* spp., random markers in random amplified polymorphic DNA (RAPD-PCR), monomorphic, polymorphic, random primers.

INTRODUCTION

The genus *Euphorbia* spp. is one of the largest and most complex genera of flowering plants. High morphological plasticity and diversity of this genus make taxonomical studies attractive for botanists. Also, this species have shown their own economic value and hence contribute to the floristic wealth of tropical and subtropical countries of the world as well as reputed for the production of valuable secondary metabolites like alkaloids, flavonoids and terpenes in nature. There are about 44 species of *Euphorbia* in Iraqi flora (Radcliff-Smith, 1980), and more than four species in University of Baghdad Campus in Jadiriyah (Zokian, 2011). Early proponents of molecular systemic claimed that molecular data were more likely to reflect the true phylogeny than morphological data, ostensibly because they reflected gene-level changes, which were thought to be less subject to convergence and parallelism than were morphological traits.

In many cases, molecular data supported the morphology of groups that were recognized on morphological grounds. More importantly, molecular data often allowed syste-matizes to choose among competing hypotheses of relationships (Judd et al., 1999). Genetic identification can be performed by examining morphological or phonotypical characteristics but such characteristics are affected by environmental conditions. However, DNA based techniques allow scanning the genome directly without being environmental affected. Today, genetic variations between species can be revealed in short time

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Table 1. The names of the random primers used in the study and their equences (Ahmadikhah and Alvi, 2009).

Number	Primer's name	Sequence 5'------ 3'
1	A07	GAAACGGGTG
2	A08	GTGACGTAGG
3	A13	CAGCACCCAC
4	C05	GATGACCGCC
5	D20	ACCCGGTCAC
6	P06	TCGGCGGTTC
7	P07	CTGCATCGTG
8	R02	GTCCTCGTGT
9	R03	ACGGTTCCAC

and easily, and the population can be examined rapidly through random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) (Sesli and Yegenoglu, 2010).

In this study, there was an attempt to identify the four species of *Euphorbia* (*Euphorbia peplus*, *Euphorbia helioscopia*, *Euphorbia granulata* and *Euphorbia hirta*) distributed at University of Baghdad Campus by using the technique of RAPD- PCR.

MATERIALS AND METHODS

Molecular weight markers

The DNA markers (Bench top PCR markers 50 to 1000 bp and 1 kb DNA ladder 250 to 10000 bp) were prepared according to the manufacturer instructions.

DNA extraction from dry seeds of *Euphorbia*

The DNA was extracted from dry seeds by using commercial kit; High Pure GMO sample preparation kit provided by Roche - Germany.

Estimation of DNA concentration by spectrophotometer

5 µl of each sample were added to 495 µl of distilled water (DW) and mixed well to determine the DNA concentration and its purity by using the spectrophotometer. A spectrophotometer was used to measure the optical density (OD) at wave length of 260 and 280 nm. An OD of 1 corresponds to approximately 50 µg/ml for double stranded DNA (Maniatis et al., 1982). The concentration of DNA was calculated according to the formula:

DNA concentration (µg/ml) = O.D 260 nm × 50 × dilution factor

The spectrophotometer was used also to estimate the DNA purity ratio according to this formula:

DNA purity ratio = O.D 260 nm / O.D 280 nm

This ratio was used to detect nucleic acid contamination in protein preparations. DNA quality can be also assessed by simply analyzing the DNA by agarose gel electrophoresis (Maniatis et al., 1982).

Agarose gel electrophoresis

Agarose gels in different concentrations were used (0.8% for extracted DNA, and 1.2% for visual checking to separate DNA fragments of RAPD product). Gels were run horizontally in 0.5X Tris-borate-EDTA (TBE) buffer. Electrophoresis buffer was added to cover the gel and run for 2 h at 5 V/cm. Agarose gels were stained with ethidium bromide 0.5 µg/ml for 20 to 30 min. DNA bands were visualized by UV transilluminator at 365 nm wavelength (Maniatis et al., 1982). A gel documentation system was used to document the observed bands.

RAPD-PCR analysis of genomic DNA of *Euphorbia* species

Randomly primers

Nine random sequence decamer primers were used, synthesis by Alpha DNA-Canada from different series (A, C, D, P and R) in a lyophilized form and were dissolved in sterile distilled water to give a final concentration of 10 pmol/µl as recommended by provider. The primers used and their sequences are listed in Table 1.

Go Taq®Green master mix (2X)

Go *Taq*®Green master mix is a ready to use mixture that contains *Taq* DNA polymerase, $MgCl_2$, pure deoxynucleotides (dNTPs), reaction buffer and two dyes (blue and yellow) that allow monitoring of progress during electrophoresis, with concentration 2X. Go *Taq*®Green master mix was provided by Promega-USA. Amplification was performed on ice in aseptic conditions in laminar air flow using 0.2 ml tight cap Eppendorf tubes. A negative control reaction in each PCR experiment was set up containing all components of the reaction without template DNA so that any contaminating DNA present in the reaction would be amplified and detected on agarose gel.

Protocol of RAPD-PCR

PCR was performed with a protocol which includes the following:

PCR mix

About 12.5 µl of the PCR ready mix (Go *Taq*®Green Master Mix) was added when the final reaction volume was 25 µl to obtain a final concentration 1X as recommended by provider and sterile distilled water was used to achieve a total volume of 25 µl after added each of primers and DNA template.

Amplification reaction

Amplification of random fragments of genomic DNA was preformed with the following master amplification reaction. RAPD-PCR master mix (final reaction volume = 25 µl).

PCR program

The amplification program was run as follows: Initial denaturartion at 94°C for 5 min with a total number of 45 cycles. Denaturation at 94°C for 1 min, annealing at 36°C for 1 min extension at 72°C for 2 min and final extension at 72°C for 10 min. Approximately, 20 µl of PCR amplified products were separated by electrophoresis in 1.2% agarose gels (2 h, 5 V/cm, 0.5 X Tris-borate buffer). Gels stained with ethidium bromide, PCR products were visualized by UV transilluminator and then were imaged by gel documentation system (Hashemi et al., 2009). The amplified products usually consist of 1 to 10 discrete bands and may increase to 15 bands, the molecular weight of RAPD-PCR products estimated by comparing with the marker 1 kb DNA ladder 250 to 10,000 bp.

RESULTS AND DISCUSSION

DNA extraction from dry seeds of *Euphorbia* species

The extraction of genomic DNA from dry seeds of *Euphorbia* spp. using commercial kit produced good quality and high purity of intact DNA to use in the RAPD-PCR analysis. The DNA yield of *E. peplus*, *E. helioscopia*, *E. granulata* and *E. hirta* were 11.5, 18.5, 9.5 and 14.0 µg per mg, respectively, of dry seeds powder, while the purity of the extracted DNA were 1.3, 1.6, 1.3 and 1.4, respectively. The integrity of the extracted DNA was checked by agarose gel 0.8%.

Molecular biological studies of plants, such as the PCR techniques, require pure DNA (Kang and Yang, 2004; Ahmadikhah and Alvi, 2009). One of the advantages of the PCR techniques is the rapid DNA analysis of many plant samples using small quantities of DNA. The DNA samples extracted from seeds were very stable and could be stored at 4 to -80°C for a long time without degradation; therefore, it could be used in further studies (Ahmadikhah and Alvi, 2009).

RAPD-PCR analysis

RAPD-PCR technique was used to reveal DNA polymorphism in DNA of the studied *Euphorbia* spp. in order to search for the sources of differences that could be used as a DNA marker represent the *Euphorbia* spp. The primers used in this study were selected randomly. Nine primers had been tested with same DNA samples under optimum conditions. The primers were classified into three groups according to results obtained. The first group gave no amplified products and this group includes (P07). Similar results were reported in different studies and a number of random primers were scored as non amplification producing primers (Al-Judy, 2004; Sujatha et al., 2005; Younan, 2010; Sesli and Yegenoglu, 2010). The second group that gave results in terms of amplification and polymorphism include A13, C05 and D20. The third group which includes A08, A07, R02, R03 and P06 primers gave amplification and polymorphism of the genomic DNA for some species, while no ampli-fication was detected with other species. The reasons of failure of these primers to amplify genomic DNA may be absence of suitable priming site for these primers on template DNA (Devos and Gale, 1992). The analysis of PCR amplified DNA fragments rely on several bases including the absence or presence of bands, and diffe-rences in molecular weight.

Levels of polymorphisms were gene-rated in this study among the four species of *Euphorbia* and also some primers generate unique bands that could be used as a DNA marker to distinguish between the local species of *Euphorbia*. In some instances, the reasons behind DNA polymorphism among samples may be due to a single base changes in genomic DNA.

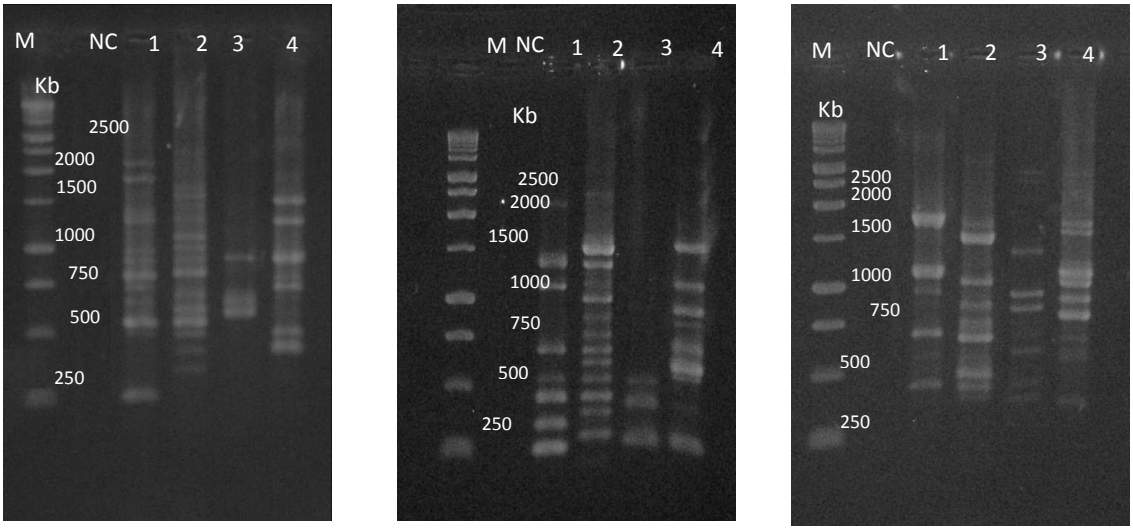
Other sources of polymorphisms may include deletions of a priming site, insertions that render priming sites too distant to support amplification, or insertions that change the size of a DNA segment without preventing its ampli-fication (Williams et al., 1990). Furthermore, it had been reported that single nucleotide changes in a primer sequence caused a complete change in the pattern of amplified DNA segments.

Primer A13

PCR results of primer A13 amplified genomic DNA of species studied of *Euphorbia* showed 33 bands as a total number of bands, distributed into 19 main bands that were polymorphic bands. The range of bands between 3 to 12 bands, *E. granulata* produced the lower number of bands only 3 bands, while *E. helioscopia* produced the highest number of bands (12 bands) as shown in Figure 1. Primer A13 generated eight unique bands, the second, third, tenth and nineteenth bands with molecular weight of about 2712, 2402, 1283 and 527 bp respectively, which distinguished *E. peplus*. The other unique bands were the first, fourth, eighth and eighteenth bands with molecular weight of about 2955, 2213, 1500 and 618 bp, respectively, which differentiated *E. helioscopia* from other species of *Euphorbia* as shown in Table 2.

Primer C05

Genomic DNA of *Euphorbia* spp. was amplified by using primer C05 and the results included a total number of 30 bands distributed into 17 main bands. Out of the 30 bands, 8 were polymorphic, ranging in molecular weight of 439 to 1569 bp, and one band were monomorphic with molecular weight of about of 690 bp (Table 3). The present monomorphic band in result of PCR reaction



Primer A13 **Primer C05** **Primer D20**

Figure 1. Agarose gel electrophoresis of RAPD-PCR reaction for random primers A13, C05 and D20 for DNA samples of Euphorbia species. Bands were fractionated by electrophoresis on a 1.2% agarose gel (2 h, 5 V/cm, 0.5X tris-borate buffer) and visualized under UV light by ethidium bromide staining. M, 1 Kb ladder; NC, negative control. Euphorbia spp.: 1, E. peplus; 2, E. helioscopia; 3, E. granulate; 4, *E. hirta*.

Table 2. The polymorphic, monomorphic and unique bands with their molecular weight for primer A13 to different *Euphorbia* species.

Number	Band molecular weight in bp	Euphorbia four spp			
		1	2	3	4
1	2955	0	1	0	0
2	2712	1	0	0	0
3	2402	1	0	0	0
4	2213	0	1	0	0
5	2630	0	1	0	1
6	1810	1	0	0	1
7	16450	1	1	0	0
8	1500	0	1	0	0
9	1400	1	0	1	1
10	1283	1	0	0	0
11	1090	1	1	0	0
12	970	1	0	0	1
13	865	1	1	1	0
14	814	0	1	1	0
15	775	1	1	0	0
16	750	0	1	0	1
17	690	0	1	0	1
18	618	0	1	0	0
19	527	1	0	0	0
Total number of band		11	12	3	6

1, Presence of bands; 0, absence of bands; , unique bands; , polymorphic bands. *Euphorbia* spp: 1, *E. peplus*; 2, *E. helioscopia;* 3, *E. granulate*; 4. *E. hirt.*

Table 3. The polymorphic, monomorphic and unique bands with their molecular weight for primer C05 to different *Euphorbia* species.

Number	Band molecular weight in bp	Euphorbia four spp			
		1	2	3	4
1	1569	1	1	0	1
2	1321	0	0	0	1
3	1225	1	0	0	0
4	1169	0	0	1	0
5	1090	1	0	0	1
6	1031	0	1	0	1
7	960	1	0	1	0
8	910	0	1	0	1
9	871	0	0	1	0
10	825	0	0	0	1
11	560	0	1	1	0
12	690	1	1	1	1
13	605	0	0	1	1
14	492	0	1	0	0
15	439	1	1	1	0
16	401	0	0	1	0
17	376	0	0	0	1
Total number of band		9	8	7	6

1, Presence of band; 0, absence of band; , unique bands; , monomorphic bands; , polymorphic bands. 1, *E. peplus*; 2, *E. helioscopia;* 3, *E. granulate*; 4, *E. hirt.*

means there was share DNA fragment in genomic of all four species of *Euphorbia.* Primer C05 produced bands in range 6 to 9 bands. *E. peplus* produced the lowest number of bands (6 bands), while *E. hirta* had the highest number of bands that produced 9 bands as shown in Figure 1. Primer C05 generated eight unique bands as shown in Table 3. *E. peplus* had one unique band which was the third band with molecular weight of about 1225 bp, as well as *E. helioscopia* distinguished by one unique band, the fourteenth band with molecular weight 492 bp, while *E. granulata* was differentiated by three unique bands, the fourth, ninth and sixteenth bands, a molecular weight of about 1169, 871 and 401 bp, respectively. Also *E. hirta* distinguished by three unique bands, the second, tenth and seventeenth bands, a molecular weight of approximately 1321, 825 and 376 bp, respectively.

Primer D20

The results of PCR reaction of primer D20 that reacted with genomic DNA of the species of *Euphorbia* showed 31 bands as a total number of bands, distributed into 16 main bands, of which 21 bands were polymorphic, the range of their molecular weight were between 241 to 2476 bp (Table 4), and there were one monomorphic band with molecular weight of about 431 bp. *E. helioscopia* produced the highest number of bands (13 bands) compared with *E. granulata* that produced three bands. This primer generated six unique bands (Table 4). The first, sixth, thirteenth bands with molecular weight of approximately 2476, 980 and 391 bp, respectively, distinguished *E. helioscopia*. The other unique bands were the second, fourteenth and sixteenth bands with molecular weight of about 2240, 339 and 250 bp, respectively, that distinguished *E. peplus* as in Figure 1.

Primers A08, A07, R02, R03 and P06

PCR results of primer A08 showed nine bands as a total number of bands for the two species *E. helioscopia* and *E. peplus*. All bands were unique, the molecular weight of these bands ranged from (500 to 2000) bp. This primer produced amplification products with range of five bands of the first species and 4 bands of the second. The genomic DNA of the two species of *E. granulata* and *E. helioscopia* were amplified by using the primers A07, R02, R03 and P06, while the genomic DNA of *E. peplus* and *E. hirta* had no amplification. The results of primer

Table 4. The polymorphic, monomorphic and unique bands with their molecular weight for primer D20 to different *Euphorbia* species.

Number	Band molecular weight in bp	Euphorbia four spp			
		1	2	3	4
1	2476	0	1	0	0
2	2240	1	0	0	0
3	1500	0	1	0	1
4	1300	1	1	0	0
5	1100	1	1	0	0
6	980	0	1	0	0
7	880	0	1	0	1
8	790	0	1	0	1
9	678	1	1	0	1
10	590	0	1	0	1
11	520	1	1	1	0
12	431	1	1	1	1
13	391	0	1	0	0
14	339	1	0	0	0
15	275	0	1	1	1
16	250	1	0	0	0
Total number of bands		7	3	13	8

1, Presence of band; 0, absence of band; , unique bands; , monomorphic bands; , polymorphic bands. *Euphorbia* spp: 1, *E. peplus*, 2. *E. helioscopia;* 3, *E. granulate*; 4. *E. hirt.*

A07 were eight bands as a total number of bands, the molecular weight of these bands ranged between 500 to 2100 bp. This primer produced amplification products with range of three bands for *E. granulata* and five bands for *E. helioscopia*. The genomic DNA of these two species was amplified by using primer R02; the results that appeared were seven bands as a total number of bands, molecular weight of bands were ranged between 545 to 1400 bp. This primer gave amplification products with range of four bands for *E. granulata* and three bands for *E. helioscopia*. While the results of primer R03 included total number of bands for the two spp., which were 12 bands, molecular weight of bands ranged between 350 to 2200 bp. This primer gave amplification products with range of nine bands for *E. granulata* and three bands for *E. helioscopia.* The primer P06 amplified genomic DNA of *E. granulata* and *E. helioscopia*. The total number were two bands, the molecular weight of the first band was about 2400 bp for *E. granulata* and the second band was about 1000 bp for *E. helioscopia*.

RAPD-PCR analyses were based on the second group of primers (A13, C05 and D20) because it gave results in terms of amplification and polymorphism for the species of *Euphorbia* as shown in Table 5. These primers produced a total of 52 main bands across the four species (Table 6). Of these 52 PCR products generated 3.85% (two bands) were monomorphic across the studied species. The remaining 50 bands (96.2% of the total products scored) were polymorphic among the species studied. This means that there is high difference among the genotypes of the species of *Euphorbia*. A total of 50 (96.2%) polymorphic bands were observed ranging from eight to 11 bands. The primer A13 gave the highest number of polymorphic bands (11), while the minimum number of polymorphic bands (8) by using C05 primer. The average number of polymorphic bands per primer among the species was 9.3. Polymorphism of each primer was calculated as the percentage of polymorphic bands to the number of total main bands produced by the designated primer. The obtained high polymorphism rate indicates a high genetic diversity. The number of bands generated by each primer varied, A13 generated maximum number of bands (33) while R02 amplified minimum number of bands (2). The variation in the number of bands amplified by different primers influenced by variable factors such as primer structure, template quantity and less number of annealing sites in the genome (Kernodle et al., 1993). Visual examination of electrophoresis gels and analysis of banding patterns confirmed that *E. peplus* and *E. helioscopia* had high degree of similarity in the pattern of DNA with most of primers compared with other species, but there were

Table 5. The species of *Euphorbia* and the primers that appeared the unique bands, the number and molecular weight of these bands.

Species name	Primer	Molecular weight of unique bands (bp)	Unique bands number
E. peplus	A13	2712	2nd
		2402	3rd
		1283	10th
	C05	527	19th
		1225	3rd
	D20	2240	2nd
		339	14th
		241	16th
E. helioscopia	A13	2955	1st
		2213	4th
		1500	8th
	C05	618	18th
		492	14th
	D20	2476	1st
		980	6th
		391	13th
E. granulata	C05	1169	4th
		871	9th
		401	16th
E.hirta	C05	1321	2nd
		825	10th
		376	17th

Table 6. Distinct characteristic of random primers included in the study: Primer's name, total number of bands, number of polymorphic bands and percentage of polymorphism in species of *Euphorbia.*

Number	Primer	Total number of main bands	Number of polymorphic bands	Polymorphism %
1	A13	19	11	84
2	C05	17	8	47
3	D20	16	9	56
Total		52	28	-

clear differences among them especially in terms of unique bands. While *E. hirta* and *E. granulata* had less similarity pattern of DNA. The RAPD assay generated specific products in all of the species studied. These may be used as DNA fingerprints for species identification. It would be of immense use for the establishment of proprietary rights and the determination of species purity.

On the other hand, RAPD markers had been useful as the first step to produce a genetic map in plants with unknown, much or less known genetic series (Sesli and Yegenoglu, 2010). On the other hand, these results confirm the isolation of the four species of *Euphorbia* from each other obviously; as well as distinction of *E. peplus* and *E. helioscopia* from the other two species *E. hirta* and *E. granulata*, and this corresponds to the morphological features of these species. These results may be applied in isolation of similar species that could not be isolated by using other qualities and characteristic features. Tian et al. (1997) showed RAPD PCR were efficient for identification of poinsettia cultivars and for determination of the genetic relationships among cultivars. Chowda-Reddy et al. (2012) showed RAPD-PCR to examine molecular variability and to select individuals with different fingerprints, and relation to recent

changes in the epidemiology of tomato leaf curl disease in South India of *Bemisia tabaci* adults from various host-plant species. Genetic variations were tested in normal and fasciated stems of *Euphorbia lactea* using RAPD-PCR fingerprints. PCR yielded different polymorphic banding patterns that were unique to each primer and distinguishable over all samples and indicated that occurrence of fasciation in *E. lactea* is an epigenetic mutation of tissues (El-Banna et al., 2013). This is one of the goals of this molecularystematic study which can be applied in such methods in the diagnosis of the rest of *Euphorbia* species existing in Iraq.

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Full Length Research Paper

Identification of phytochemical components of aloe plantlets by gas chromatography-mass spectrometry

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Aloe vera **plants were collected from Blochestan, Iran and were transferred to tissue culture laboratory. Shoot tip explants were inoculated on solid MS medium supplemented with 0.5 mgl^{-1} benzyl adenine + 0.5 mgl^{-1} α-naphthalene acetic acid and sub-cultured on the same medium for plantlet production and propagation once every four weeks. After plantlets production, extracts of *A. vera* plantlet were analyzed by gas chromatography-mass spectrometry (GC-MS). According to the results, 26 phytochemical compounds were identified. Results indicate that these compounds of micropropagated plantlets are similar to the phytochemical compounds identified by other researchers in aloe plants. With attention on the obtained results of GC-MS analysis, the obtained compounds of micropropagated plantlets did not vary in relation to aloe plants. These results also indicate that the use of propagated plantlets by tissue culture to produce and extract phytochemical compounds is useful and efficient, as was observed and expected. So, we can use this method (tissue culture) instead of aloe cultivation which is limited in some regions of the world.**

Key words: Aloe medicinal plant, phytochemical components, micropropagation, tissue culture, gas chromatography-mass spectrometry (GC-MS) analysis.

INTRODUCTION

Aloe vera is a medicinal, cosmetic and ornamental plant. The genus Aloe is a perennial succulent herb growing in tropical and subtropical parts of the world. Therefore, aloe cultivation is limited in these regions. There are over 300 species of aloe; most of them are native to South Africa, Madagascar and Arabia.

The different species have somewhat different concentrations of active ingredients (Yagi et al., 1998; Van Wyk et al., 1995). At least, a quarter of Aloe genera is valued for traditional medicine (Grace et al., 2009), while a small number is wild harvested or cultivated for natural products prepared from the bitter leaf exudate or gel-like leaf mesophyll; *A. vera* is commonly cultivated and supports a global natural products industry. Today, *A. vera* gel is an active ingredient in hundreds of skin lotions, sun blocks and cosmetics (Grindlay et al., 1986). Aloe gel is 99% water with a pH of 4.5 and is a common ingredient in many non-prescription skin salves. Aloe extracts have been used to treat canker sores, stomach ulcers and even AIDS. The gel contains an emollient polysaccharide, glucomannan, which is a good moisturizer utilized in many cosmetics (Henry, 1979). Acemannan, the major carbohydrate fraction in the gel demonstrates antineoplastic and antiviral effects (Mc Daniel et al., 1990). The gel also contains bradykininase, an anti-inflammatory agent, which prevents itching, and salicylic acid as well as other antiprostaglandin compounds that relieve inflammation (Yagi et al., 1982). Other important pharmacological activities of *A. vera* are anti-diabetic (Rajasekaran et al., 2006), antiseptic (Capasso et al., 1998), anti-tumor (Winter et al., 1981), and wound and burn healing effect (Heggers et al.,

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Abbreviations: GC-MS, Gas chromatography-mass spectrometry; **MS,** Murashige and Skoog medium.

Figure 1. *Aloe vera* plant.

1993). The sticky liquid latex is derived from the yellowish-green pericyclic tubules that line the leaf (rind); this is the part that yields laxative anthraquinones. The leaf lining (latex, resin or sap) contains anthraquinone glycolsides (aloe-emodin and barbaloin) which are potent stimulant laxatives.

Sexual reproduction by seeds due to male sterility in aloe plants is almost not effective and vegetative propagation through lateral shoots or lateral buds is only possible during growing seasons (Nayanakantha et al., 2010), and is slow and very expensive for commercial plant production (Meyer and Staden, 1991). To overcome slow propagation rate, micro propagation will be a very useful technique for mass production of aloe.

A. vera has been cultured *in vitro* by various researchers (Natali et al., 1990; Roy and Sarkar, 1991; Abrie and Staden, 2001). The technique of tissue and organ culture is used for rapid multiplication of plants, for genetic improvement of crops, for obtaining disease-free clones and for preserving valuable germplasm. One of the major applications of plant tissue culture is micropropagation or rapid multiplication. As compared to conventional propagation, micropropagation has the advantage of allowing rapid propagation in limited time and space.

Gas chromatography-mass spectrometry (GC-MS) is a method that combines the features of gas liquid chromatography and mass spectrometry to identify different substances within a test sample. GC-MS can provide meaningful information for components that are volatile, non-ionic, thermally stable and have relatively low molecular weight.

In this present study, we used micropropagated plantlets of *A. vera* for evaluation of phytochemical components by GC-MS analysis.

MATERIALS AND METHODS

A. vera plants (Figure 1) were collected from Blochestan farmland in Iran and were transferred to Tissue Culture Laboratory of Karaj Agricultural Biotechnology Research Institute in September 2010. Shoot tip explants containing one to two buds were cut and washed with tap water for 10 min, and after surface sterilization using 2% (w/v) NaOCl for 20 min, they were thoroughly rinsed with sterile water. The explants were thoroughly washed with sterile double distilled water for four to five times to remove any trace of the sterilant. Then, the explants were inoculated on solid Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium supplemented with 0.5 mgl^{-1} benzyl adenine + 0.5 mgl^{-1} α-naphthalene acetic acids into jars (250 ml capacity) containing 40 ml of the above-mentioned medium. Samples were sub-cultured every four weeks once on the same medium for plantlet production and propagation (Figure 2).

Preparation of plant extract

The micropropagated *A. vera* plantlets were washed with distilled water and were kept in-room temperature to be dried by air. Dried

Figure 2. Micropropagated plantlets of *A. vera*.

plantlets were crushed to the small pieces and were powdered and kept in polythene bags for further use. Aqueous extract of the studied samples were used to carry out the qualitative and quantitative analysis using standard procedures to identify the phytochemical components as described by Sofowara (1993) and Trease and Evans (1989).

Extracts of *A. vera* plantlets were analyzed by GC-MS. GC analysis was performed using a Hewlett-Packard 6890 chromatograph equipped with a flame ionization detector and injector MS transfer line with temperature of 280°C, respectively. A fused silica capillary column Hp- 5ms (5% phenyl : 95% dimethyl siloxane 30 M × 0.25 mm film thickness 0. 32 Lm) was used. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C. The carrier gas helium was at a flow rate of 1 ml/min. GC-MS analyses were carried out on an Agilent Technologies Network mass spectrometer (model 5973) coupled to H.P. gas chromatograph (model 6890) equipped with NBS 75K Library Software database. The capillary column and GC conditions were as described above. Mass spectra were taken at 70 eV; the scanning rate was 1 scan/s and the run time was 90 min. Compound identification was accomplished by comparing the GC relative retention times and mass spectra to those of authentic substances analyzed under the same conditions, by their retention indices (RI) and by comparison with reference components.

RESULTS AND DISCUSSION

The utilization of GC-MS was effective and useful for the identification of the bioactive compounds in *A. vera*. According to the results, 26 bio-active phytochemical compounds were identified in the GC-MS analysis of *A. vera* plantlets. The identification of phytochemical compounds is based on the peak area, molecular weight and molecular formula (Table 1).

Results indicate that these compounds of micropropagated plantlets are similar to the phytochemical compounds identified by other researchers in aloe plants (Sathyaprabha et al., 2010; Lakshmi et al., 2011). 10 compounds with biological activities were found in *Aloe vera*. The main compounds include oleic acid (14.49), 11,14-eicosadienoic acid, methyl ester (2.71), n-hexadecanoic acid (20.41), 1,2-benzenedicarboxylic acid, butyloctyl ester (2.28), hexadecanoic acid, methyl ester (1.45), tetradecanoic acid (1.03), (4,7-dinitronaphthalen-1-yl)-(4-methoxyphenyl)diazene (0.09), 1-heptanol, 2-propyl- (3.77), 1,2-benzenedicarboxylic acid, diisooctyl ester (13.56) and squalene (6.57). These compounds of *A. vera* were shown to have the activity of anticancer, antimicrobial, etc. These results also indicate that the use of propagated plantlets by tissue culture to produce and extract phytochemical compounds is useful and efficient, as was observed and expected. So, we can use this method (tissue culture) instead of aloe cultivation which is limited in some regions of the world for production and extraction of phytochemical compounds.

The composition of identified active compounds in *A. vera* is the subject of future research studies. With attention on the obtained results of GC-MS analysis, the obtained compounds of micropropagated plantlets do not vary in relation to aloe plants, and the bioactive phytochemical compounds have not changed within micropropagated plantlets in relation to wild plant (Figures 3 and 4). With regards to the variations of environmental and growth conditions, the phytochemical profiles of individual plants changes. Wild plants may produce secondary metabolites, which have no apparent

Table 1. Identified components of *A. vera* plantlet by GC-MS.

RT	Name of the compound	Molecular Formula	Molecular Weight	Peak area (%)
3.06	p-Xylene	C_8H_{10}	106	3.13
3.78	1,5-Heptadien4-one,3,3,6-trimethyle-	$C_{10}H_{16}O$	152	1.69
7.03	1- Heptanol, 2- propyl-	$C_{10}H_{22}O$	158	3.77
8.25	Tridecane	$C_{13}H_{28}$	184	0.1
9.59	7- Tetradecane, (z)-	C14H28	196	0.17
10.87	Tetradecane	$C_{14}H_{30}$	198	0.32
12.14	Hexadecane	$C_{16}H_{34}$	226	0.38
13.64	12,15-Octadecadiynoic acid,methyle ester	$C_{19}H_{30}O_2$	290	0.18
13.96	(4,7-Dinitronaphthalen-1-yl)-(4-methoxyphenyl)diazene	$C_{17}H_{12}N_4O_5$	352	0.09
14.44	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	1.03
15.87	Octadecane, 3- ethyl-5-(2-ethylbutyl)-	$C_{26}H_{54}$	366	0.21
17.28	Undecane	$C_{11}H_{24}$	156	0.45
17.42	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390	13.56
19.38	9-Octadecenoic acid, (2-phenyl-1,3dioxolan-4-yl)methyle ester.cis-	$C_{28}H_{44}O_4$	444	3.04
20.74	9,12,15- Octadecatrienoic acid, 2-((trimethylsilyl)oxy)-1-(((trimethylsilyl)oxy)methyl) ethyl ester, (ZZZ)-	$C_{27}H_{52}O_4Si_2$	496	2.08
20.40	Oleic acid	$C_{18}H_{34}O_2$	282	14.49
24.68	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	1.45
25.94	1,2-Benzenedicarboxylic acid, butyloctyl ester	$C_{20}H_{30}O_4$	334	2.28
27.26	n- Hexadecanoic acid	$C_{16}H_{32}O_2$	256	20.41
28.71	11,14-Eicosadienoic acid, methyl ester	$C_{21}H_{38}O_2$	322	2.71
29.11	1-Monolinoleoylglycerol trimethylsilyl ether	$C_{27}H_{54}O_4Si_2$	498	2.63
30.48	Eicosane	$C_{20}H_{42}$	282	3.36
31.20	Heptacosane	$C_{27}H_{56}$	380	6.08
32.77	Octacosane	$C_{28}H_{58}$	394	9.52
34	Squalene	$C_{30}H_{50}$	410	6.57
35.43	Hentriacontane	$C_{31}H_{64}$	436	8.14

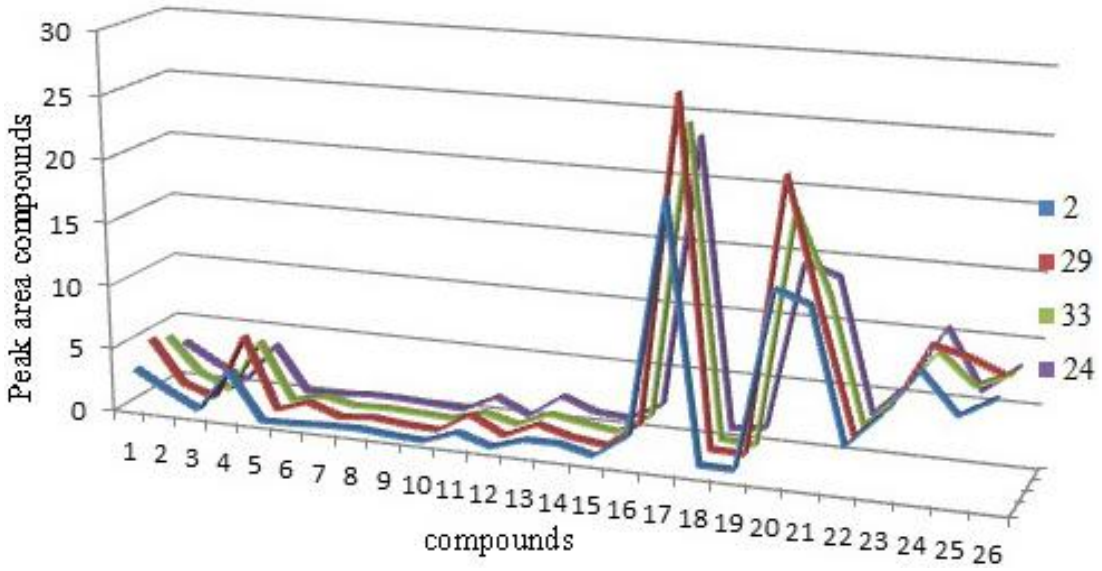

Figure 3. GC-MS graph of *A. vera* plantlets (2, 29, 33 and 24 accessions).

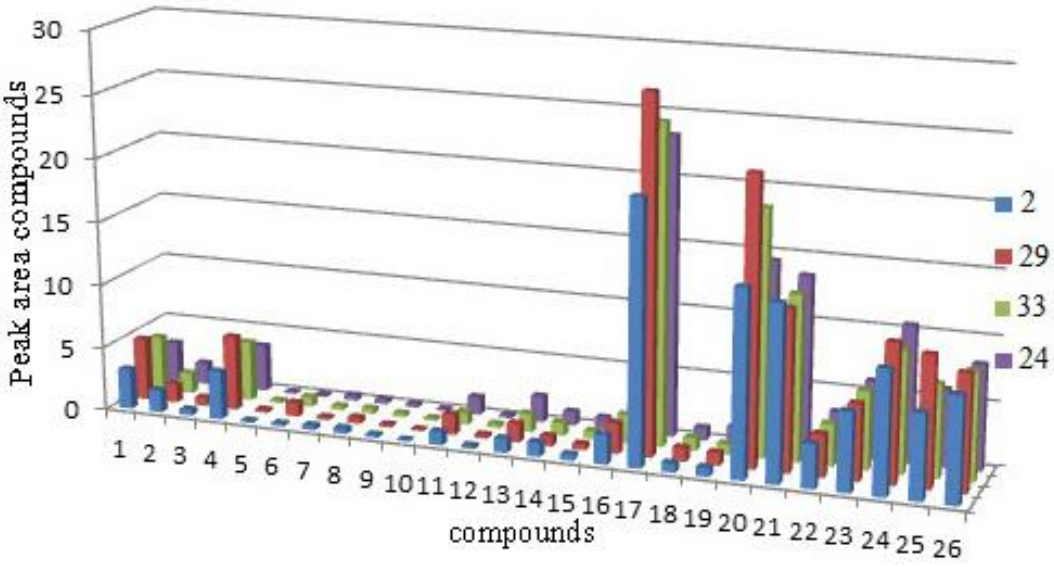

Figure 4. GC-MS graph of *A. vera* plantlet (2, 29, 33 and 24 accessions).

role in primary plant growth or development processes. These molecules are often unique in plants of a single species and are increased during times of high stress such as drought, fire and bacterial infection stresses in micropropagated plantlets.

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Full Length Research Paper

The role of *Nardostachys jatamansi* against doxorubicin-induced toxicity in rats

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This investigation elucidated the role of free radicals in doxorubicin-induced toxicity and protection by *Nardostachys jatamansi (NJ)*. Adult male albino wistar rats were administered with doxorubicin (15 mg/kg; i.p.) and *NJ* (500 mg/kg, orally) for seven days. At the end of the experiment, following decapitation, heart and liver tissue samples were taken for histological examination, determination of malondialdehyde (MDA), glutathione (GSH) and myeloperoxidase (MPO) activity. In addition, proinflammatory cytokine (TNF-α) was assayed in plasma samples. The results reveal that doxorubicin caused a significant decrease in GSH level, significant increases in MDA level and MPO activity. Similarly, plasma cytokine level was elevated in doxorubicin group compared with the control group. On the other hand *NJ* pretreatment reversed all these biochemical indices. The results demonstrate that *NJ* extract, by balancing the oxidant-antioxidant status and inhibiting the generation of proinflammatory cytokine, protects against doxorubicin-induced oxidative organ injury.

Key words: *Nardostachys jatamansi*, doxorubicin, cytokine, glutathione, malondialdehyde, myeloperoxidase.

INTRODUCTION

Doxorubicin (DOX) is a quinine-containing anticancer antibiotic and widely used to treat different types of human neoplastic disease such as hematopoietic, lymphoblastic and solid tumors (Hassanpour et al., 2010). However, its clinical use is limited on account of its toxicity. DOX cytotoxic effects have been associated with reactive oxygen species (ROS) generated during drug metabolism. Several *in vivo* and *in vitro* studies have demonstrated that reactive oxygen metabolites including free radical species, superoxide anion (O_2−•), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) are important mediators of tissue injury (Mohamad et al., 2009). The cellular and biochemical changes involved in this process have been demonstrated. One-electron reduction of DOX leads to formation of the corresponding semiquinone free radical. In the presence of oxygen, this free radical rapidly donates its electron to oxygen to generate superoxide anion (O_2 •). The dismutation of superoxide yields hydrogen peroxide (H_2O_2). Under biological conditions, the anthracycline semiquinone or reduced metal ions such as iron reductively cleaves hydrogen peroxide to produce the hydroxyl radical which is the most reactive and destructive chemical species ever known. This ultimately leads to lipid peroxidation, causing irreversible damage of membrane structure and function (Giri et al., 2004).

An extract of the rhizomes of *Nardostachys jatamansi* mainly composed of sesquiterpenes, lignans, neolignans, alkaloids and coumarins, has been shown to exhibit a variety of pharmacological actions (Subashini et al., 2006). *N. jatamansi* extract *(NJ)* has been reported to be a potent free radical scavenger and an antioxidant. The extract of rhizome and its ingredients provide protection in myocardial and oxidative injury (Subashini et al., 2006; Ali et al., 2000). The antioxidant effects of *NJ* in doxorubicin toxicity, was shown previously (Lyle et al., 2009).

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Accordingly, the present study aimed to investigate the possible protective effect of *NJ* against doxorubicin induced oxidative damage of the heart and liver tissues by using biochemical approaches, such as the measurement of malondialdehyde (MDA), glutathione (GSH) levels and myeloperoxidase (MPO) activity as well as by the histological analysis of tissue injury.

MATERIALS AND METHODS

Plant material and decoction preparation

Roots of *N. jatamansi* De Jones (Valerianaceae) were identified and authenticated by Dr. Sasikala Ethirajulu, (Research Officer, Botany) of the Central Institute for Siddha (CRIS), Arumbakkam, Chennai-600 101. Clean roots were air dried and powdered to prepare the alcoholic extract as earlier described (Prabhu et al., 1994). 1 kg of moderately powdered rhizomes of *Jatamansi* was extracted by refluxing with 95% ethyl alcohol in Soxhlet extractor for 6-8 h. The extract was evaporated to dryness under reduced pressure and temperature using rotatory vacuum evaporator, and dried residue was stored at 4°C. The yield of dry extract from crude powder of *Jatamansi* was 10%. The dried ethanolic extract was suspended in distilled water which was then administered to rats orally at an optimum dosage of 500 mg/kg body wt. This particular dosage was fixed after trying out different doses for different days in the same set of rats (Subashini et al., 2006).

Test animals

Adult male Albino rats of Wistar strain weighing about 120 - 130 g were used in this study. They were maintained in clean, sterile, polypropylene cages and fed with commercial pelleted rat chow (M/s. Hindustan Lever Ltd., Bangalore, India), water *ad libitum.* Experimental animals were handled according to the University and institutional legislation, regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justices and Empowerment, Government of India (IAEC No.01/007/06).

Induction of experimental toxicity and treatment

The following groups of animals were used. The rats were divided into four groups (n=6 in each group): Group I: Rats served as the control; Group II: Rats were given doxorubicin (15 mg / kg body wt., intraperitoneally) on day 7 (Nagi and Mansour, 2000; Abdel-Wahab, 2003), Group III: Rats were pretreated orally with *NJ* extract (500 mg /kg body wt., orally for 7days), Group IV: Rats were pretreated orally with *NJ* extract at the above mentioned dosage for seven days and were given doxorubicin (15 mg / kg body wt., intraperitoneally) on day 7. The animals were sacrificed after 48 h of experimental period. The blood was collected and plasma separated by centrifugation at 2500 *g*. The heart and liver tissue were dissected out and washed in ice-cold saline. A portion of the heart and liver tissue were homogenized in 0.1 M Tris-HCl buffer, pH 7.4 and used for various biochemical experiments.

Analytical procedure

MPO activity was measured in tissues by a procedure similar to that documented by Vaghasiya et al., 2010, MDA formation was estimated by the method of Slater and Sawyer (1971), reduced glutathione was determined by the method of Moron et al. (1979), and plasma levels of TNF-α was determined by using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions.

Histopathology

A small portion of the liver and heart tissue from the control and experimental animals were fixed in 10% neutral buffered formalin and processed by standard procedure for paraffin embedding and serial sections were cut (5 µM). The sections were stained with hematoxylin and eosin dyes.

Statistical analysis

The values were expressed as mean ± SD for six rats in each group. All data's were analyzed with SPSS/10 student software. Hypothesis testing method included one way analysis of variance (ANOVA) followed by post hoc testing performed for inter group comparison with least significant difference (LSD) test. The values were expressed as mean ± SD, $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Doxorubicin exposure is associated with several toxic manifestations in humans and laboratory animals, in which heart, liver and kidney being the most sensitive (Mohamed et al., 2004). The results of the present study indicate that administration of DOX in a dose of 15 mg/kg, i.p. significantly elevated the cardiac and liver tissue content of MDA, and a significant decrease of GSH level. Studying the toxic effects of doxorubicin in mice previously, demonstrated that when administered chronically, doxorubicin caused multiorgan damage by increasing lipid peroxidation of the tissues (Hrelia et al., 2002). Furthermore when *Gingko biloba* extract was administered concomitantly with doxorubicin, this damage was prevented, suggesting that doxorubicin caused oxidative injury and that antioxidative agents could be beneficial against doxorubicin toxicity (Naidu et al., 2002).

As a free radical generating system, lipid peroxidation has been suggested to be closely related to oxidant-induced tissue damage, and MDA is a good indicator of the degree of lipid peroxidation (Vuchetich et al., 1996). In this study, it was observed that doxorubicin administration resulted in a significant increase in MDA levels in tissues (heart and liver) compared with the control animals.

However, these elevations were significantly reversed with *NJ* administration ($p < 0.05$) (Tables 1 and 2). The association between elevated cardiac and liver tissue content of MDA and lowered cardiac content of GSH, found in this study, strongly proves the oxidative damage caused by DOX. This observation has been supported by previous findings (Yilmaz et al., 2006; Injac et al., 2008). As shown in this study, the antioxidant *NJ* treatment significantly inhibited MDA production with a concomitant

Table 1. Effect of *NJ* on cardiac content of reduced glutathione (GSH) and melandialdhde (MDA) following doxorubicin administration

Parameter	GSH (µmol/g tissue)	MDA (nmol/g protein)
Control	4.5 ± 0.05	51.3 ± 1.55
DOX	2.8 ± 0.03*	90.0 ± 1.70*
NJ treated	4.7 ± 0.07	45.6 ± 1.04
NJ + DOX treated group	3.2 ± 0.09*	48.4 ± 1.39*

Data's are expressed as mean ± SD; n=*6*. One way ANOVA was followed by post hoc test LSD, **P < 0.05*, (Comparisons: control vs DOX induced group; DOX induced group vs *NJ* + DOX induced group).

Table 2. Effect of *NJ* on Liver tissue content of reduced glutathione (GSH) and melandialdhde (MDA) following doxorubicin administration.

Parameter	GSH (µmol/g tissue)	MDA (nmol/g protein)
Control	1.96 ± 0.12	48.36 ± 1.91
DOX	0.83 ± 0.01*	81.05 ± 3.72*
NJ treated	1.98 ± 0.18	50.61 ± 2.00
NJ + DOX treated group	1.45 ± 0.17*	60.43 ± 3.80*

Data's are expressed as mean ± SD; n=*6*. One way ANOVA was followed by post hoc test LSD, **P < 0.05*, (Comparisons: control vs DOX induced group; DOX induced group vs *NJ* + DOX induced group).

Table 3. The effects of doxorubicin and *Nardostachys jatamansi* extract *(NJ)* treatment on the myeloperoxidase activity (MPO) of the heart and liver tissues of groups.

Parameter	MPO in heart (U/g)	MPO in liver (U/g)
Control	0.62 ± 0.03	12.36 ± 1.41
DOX	6.34 ± 0.20*	28.05 ± 2.90*
NJ treated	0.60 ± 0.08	11.93 ± 1.82
NJ + DOX treated group	3.98 ± 0.71*	15.70 ± 1.87*

Data's are expressed as mean ± SD; n=*6*. One way ANOVA was followed by post hoc test LSD, **P < 0.05*, (Comparisons: control vs. DOX induced group; DOX induced group vs. *NJ* + DOX induced group).

replenishment of tissue GSH content, implying a reduction in lipid peroxidation and cellular injury, which protects the heart and liver tissues against doxorubicin-induced oxidative damage. An increase in MPO activity due to doxorubicin may cause inflammation and damage in the organs. MPO activity, which is an indicator of tissue neutrophil infiltration, was increased in all the studied tissues due to doxorubicin ($p < 0.05$) and *NJ* significantly reduced the tissue MPO activity ($p < 0.05$) (Table 3).

It has been suggested that an increase in lipid peroxidation may be due partly to the free radicals generated by neutrophils because activated neutrophils are known to induce tissue injury through the production and release of reactive oxygen metabolites and cytotoxic proteins into the extracellular fluid. When neutrophils are stimulated by various stimulants, myeloperoxidase, as well as other tissue-damaging substances, is released from the cells (Kettle and Winterbourn, 1997).

On the other hand, the proinflammatory cytokine, TNF-alpha, was found to be significantly increased, also verifying that doxorubicin toxicity is closely related with inflammatory mechanisms and oxidative damage (Figure 1). These observations have been supported by previous findings (Naiyra et al., 2010).

Since *NJ* treatment significantly decreased these cytokines and prevented the infiltration of neutrophils into the damaged tissue, the results suggest that the protective effects of *NJ* were mediated in part by blocking plasma cytokines and tissue neutrophil infiltration. This might also result in reduced lipid peroxidation and less accumulation

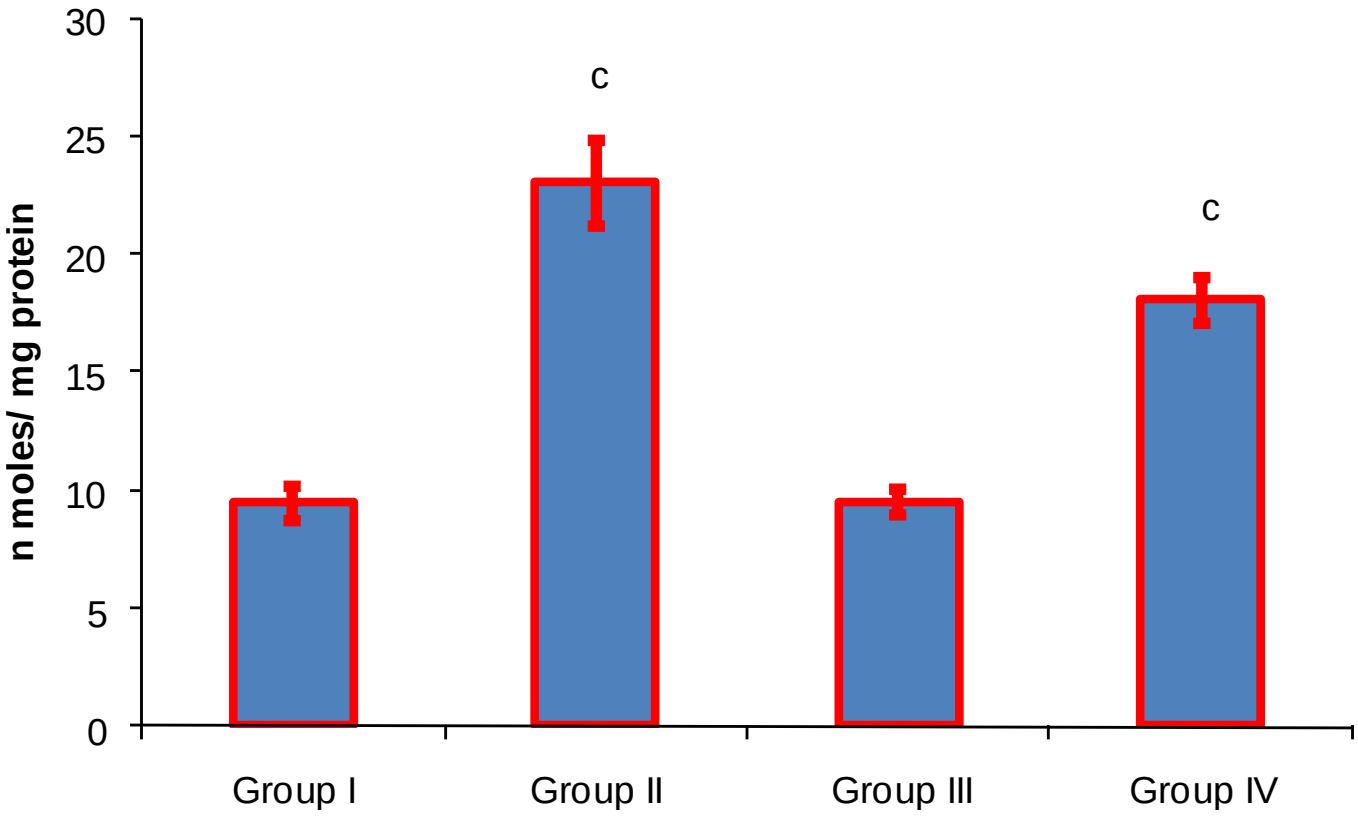

Figure 1. The effects of doxorubicin and *Nardostachys jatamansi extract (NJ)* treatment on the plasma levels of TNF-α. Group 1: Control; Group II, DOX induced; Group III, *NJ* treated; Group IV, *NJ* + DOX treated; Units: pg/ml. Values are expressed as mean ± SD; *n=6*. One way ANOVA was followed by post hoc test LSD. $^{c}P < 0.05$, (Comparisons: group I vs. group II; group II vs. group IV).

of MDA since activation of neutrophils might lead to the generation of oxygen reactive metabolites (Kettle and Winterbourn, 1997).

The histological results also verified the doxorubicin induced oxidative injury, as demonstrated by biological parameters. Figure 3 A-D shows the histological pictures of the liver cells of experimental animals. The histological examination of control rats showed normal architecture when viewed under the microscope.

The liver cells from the rats pretreated with *NJ* alone also revealed normal architecture when viewed under the microscope. Histological examination of hepatocytes from DOX treated rats showed vacuolar degenerative changes, focal necrosis, and increased kupfur cell activity, was also observed. In rats pretreated with *NJ* and administered with DOX, there was reduced overall degeneration where mild hepatocellular degeneration persisted.

Figure 2 A-D shows the histological pictures of the cardiac cells of experimental animals. The histological examination of the control rats showed normal architecture when viewed under the microscope.

The cardiac cells from the rats pretreated with *NJ* alone also revealed normal architecture when viewed under the microscope. Histological examination of cardiac cells from DOX treated rats showed myocardial coagulative necrosis, vascular dilatation and inflammatory cellular infiltration and fibrosis. Rats pretreated with *NJ* and administered with DOX, showed lesser cellular infiltration, and less myocardial necrosis than DOX-treated rats.

In the present study, increases in lipid peroxidation and myeloperoxidase activity due to the toxic effects of doxorubicin were accompanied by significant reductions in glutathione levels of the hepatic and cardiac tissues, suggesting the presence of oxidative tissue damage.

Furthermore, the elevated plasma level of the cytokine TNF-α and the histological analyses demonstrated the severity of the doxorubicin induced systemic inflammatory response. *NJ* as an antioxidant agent, amelio-rated the oxidative injury and inhibited the cytokine release.

In conclusion, the protective effects of *NJ* can be attributed, at least in part, due to its free radicals scavenging capability, ability to inhibit neutrophil infiltration and to regulate the generation of inflammatory mediators, suggesting a future role in the treatment of multiorgan damage due to drug or chemical toxicities. On the basis of our findings, it may be worthy to suggest the concomitant administration of *NJ* with DOX cancer chemotherapy.

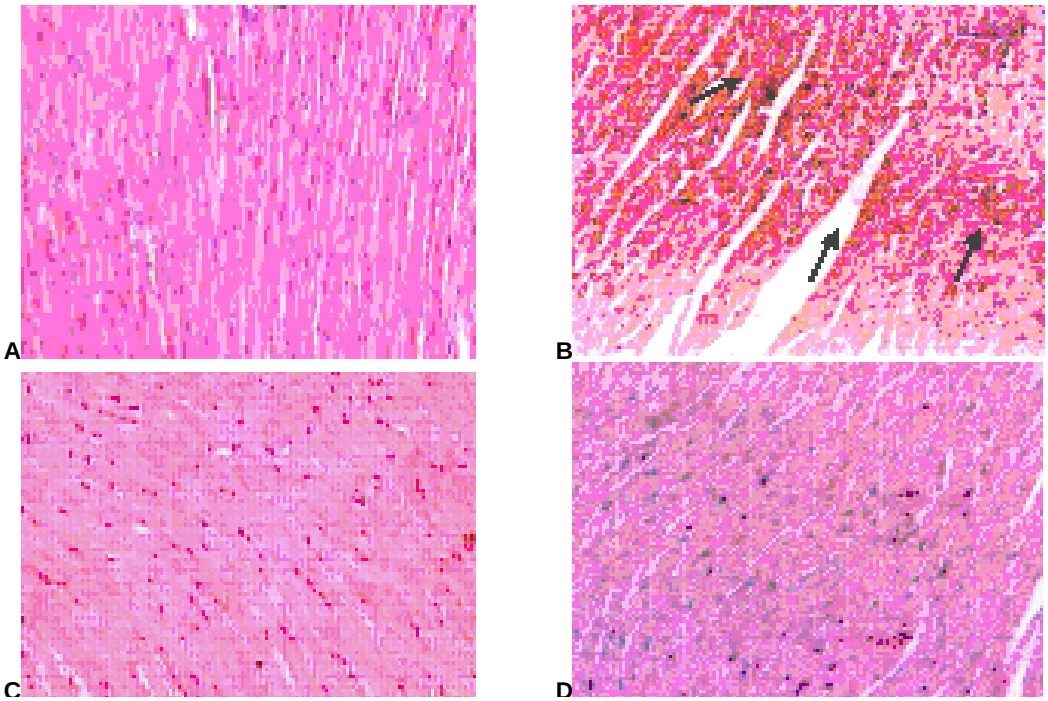

Figure 2. Histopathological changes of cardiac muscle tissue. **A.** Cardiac muscle tissue of control rats showing normal architecture H&E. **B.** Cardiac muscle tissue of DOX treated rats H&E. **C.** Cardiac muscle tissue of *NJ* treated rats showing apparently normal architecture H&E. **D.** Cardiac muscle tissue of rats treated with DOX followed by *NJ* H&E.

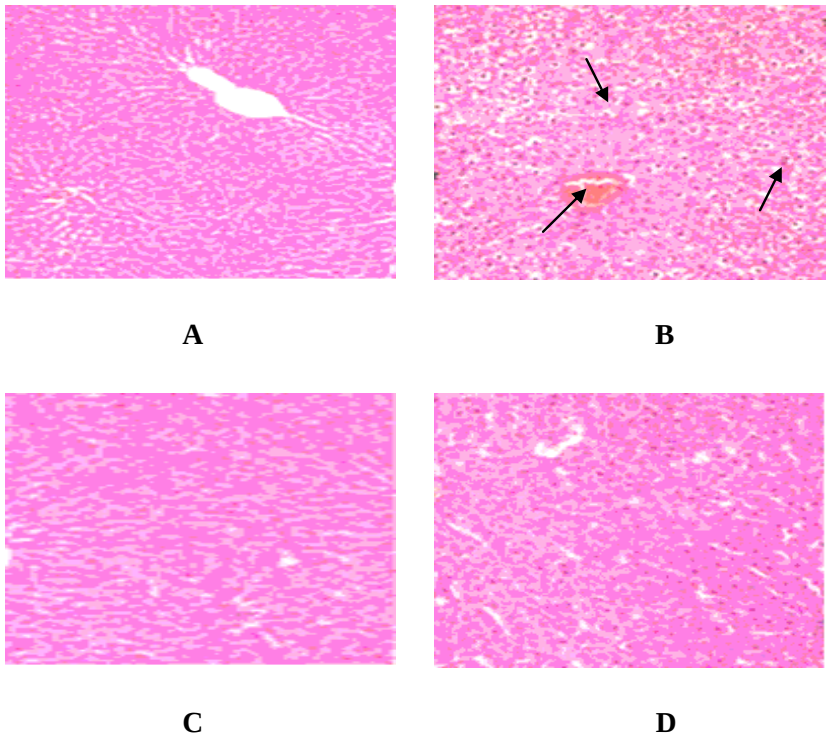

Figure 3. Histopathological changes of liver tissue. **A.** Liver of control rats showing normal architecture H&E. **B.** Liver of DOX treated rats H&E; **C.** Liver of *NJ* treated rats showing apparently normal architecture H&E. **D.** Liver of rats treated with DOX followed by *NJ* H&E.

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Full Length Research Paper

Effect of potassium simplex optimization medium (KSOM) and embryo screening on the production of human lactoferrin transgenic cloned dairy goats

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In this study, we produced cloned transgenic dairy goat based on dairy goat ear skin fibroblast as donor cells for nuclear transfer (NT), which were modified by human lactoferrin (*hLF*) gene. The developmental competence of NT embryos was compared with either between different embryo culture medium, potassium simplex optimization medium (KSOM) and tissue culture medium (TCM 199), or different classification of NT embryos (48 h after fusion). First we cultured NT embryos to cleavage stage (48 h after fusion) by TCM 199 supplemented with 1 mg/ml bovine serum albumin BSA and KSOM, then used TCM 199 supplemented with 10% FBS to culture them to blastula stage. The results show that the NT embryos in KSOM (19.5%) were superior to TCM 199 (10.6%) in blastulation. In the second experiment, we found that the growth rate of NT embryos (48 h after fusion) was different, then we divided them into four groups: 2-cell, 3- to 4-cell, 5- to 8-cell and >8-cell in stereo microscope and cultured them *in vitro* respectively. The results show day-2 embryos at 3-4cell and 5-8cell stage (31.9 and 28.2%, $P < 0.05$) had higher blastocyst formation rates than those at both 2-cell (9.1%) and >8-cell (8.3%) stage, and finally three healthy cloned transgenic goat were successfully produced using 3-8 cell embryos at Day-2 (82%). Using Hoechst 33342 staining, we also found that the >8 cells embryos at Day-2 demonstrated higher frequency of fragmentation, which may be the one cause of the low blastocyst formation rate. This study therefore demonstrates that KSOM medium could be selected as the early embryo culture medium, and 3-8 cell embryos at day-2 (48 h after fusion) may be the suitable embryos for transplantation, which could reduce the nuclei fragmentation and result in good quality blastocysts that may also enhance the efficiency of transgenic cloned dairy goats production, as well as decrease the economic loss due to embryonic mortality when embryos are transferred to synchronized recipients.

Key words: Nuclear transfer, KSOM, transgenic, human lactoferrin, dairy goat.

INTRODUCTION

Since the first report of a live mammal produced by nuclear transfer (NT) of a cultured cell line in 1996 (Campbell et al., 1996), cloned mammals have been produced successfully in sheep (Wilmut et al., 1997), cattle (Kato et al., 1998), goat (Baguisi et al., 1999) and pig (Polejaeva et al., 2000). An effective system for genetic modification and somatic cell nuclear transfer (SCNT) to produce transgenic animals would find application in the fields of agriculture, biotechnology and human medicine. Although, there are reports of live transgenic cloned offspring produced by SCNT in goats, the efficiency of cloned animal production is still less than

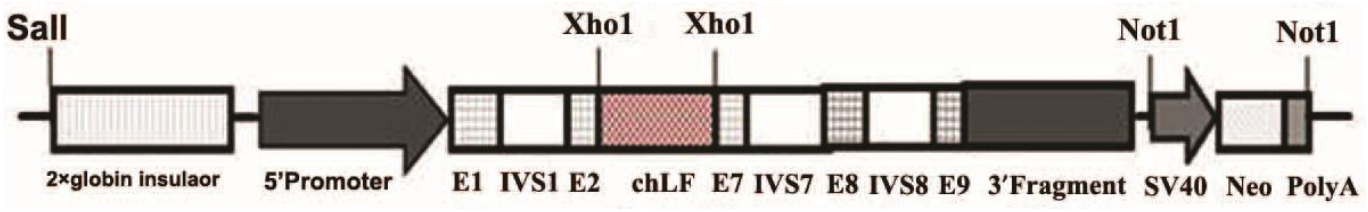

Figure 1. Schematic diagram of the pBC1-*hLF*-Neo mammary gland-specific vector.

satisfactory. The main barriers for transgenic animal production are culture conditions and nuclear transfer protocols.

Numerous studies have been performed to improve *in vitro* developmental competence of goat SCNT embryos using different fusion/activation protocols or culture medium by adding a variety of serum factors or different donor cells (Apimeteetumrong et al., 2004; Melican et al., 2005; Lan et al., 2006; Guo et al., 2009, 2010). However, the development rate of transgenic NT embryo currently remains low, indicating that embryo culture medium cannot provide the necessary ingredients for the development of the *in vitro* embryo. Potassium simplex optimization medium (KSOM) is an optimized medium (Lawitts and Biggers, 1993) that has achieved good results in mouse, rat and rabbit embryos. However, there has been no report of the production of transgenic goat embryos in livestock using KSOM medium.

Human Lactoferrin (*hLF*) is an iron-binding protein of the transferring family (Hyvonen et al., 2006). The molecular mass of LF is approximately 80 kDa. The N- and C-terminus of LF fold into two globular lobes, and each lobe contains one iron-binding site which can bind two ferric ions reversible (Anderson et al., 1989). This protein has been found to have a number of biological functions, including antimicrobial, anticancer, antioxidant and immunomodulatory effects (Yen et al., 2011). Several studies indicate that *hLF* binds to DNA (He and Furmanski, 1995), and Baumrucker et al. (2006) found that exogenous application of *hLF* stimulates the expression of a retinoic acid DR5 luciferase reporter construct in a mammary cell line. *hLF* is produced by various exocrine glands in the human body and is abundantly present in milk and colostrums (Amini and Nair, 2011). However, there is a potential safety problem and limitations on human lactoferrin (*hLF*) production by purification from human body. Hence, it is much more practical and effective to produce *hLF* from the mammary gland of transgenic animals.

The objective of the present study was to establish a system to produce transgenic goats harboring the *hLF* gene by using NT. We also evaluated the effect of the KSOM and tissue culture medium (TCM199) on the developmental ability of transgenic goat embryo by NT.

MATERIALS AND METHODS

Chemicals

Unless otherwise indicated, all chemicals used in this study were obtained from Sigma-Aldrich Company (St. Louis, MO, USA) and the media from Gibco Invitrogen Corporation (Grand Island, NY, USA).

Transgene construct and transfection

Human *hLF* genomic cDNA was cloned using the goat beta-casein promoter as a regulatory controller. The neomycin gene was isolated from the pcDNA 3.1 vector and cloned into the goat b-casein promoter/*hLF* vector (Figure 1). The goat β-casein promoter/*hLF* vector linearized with Lipofectamine LTX (Invitrogen, Grand Island, NY, USA) was used for *hLF* gene transfection. The dairy goat-derived fetal fibroblasts were seeded in a 6-well dish at a concentration of 3×10^3 cells per well and incubated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) for 48 h. The cells were transfected with linearized plasmid DNA according to the protocol described by the manufacturer. The transfected cells were split into100-mm plates. After 48 h of culture, colonies were selected with 500 mg/ml of G418 (Invitrogen) for 14 days. Well-proliferated colonies were isolated and screened for the presence of the transgene. The colonies were then frozen until use for nuclear transfer.

Collection and *in vitro* maturation of goat oocytes

Goat ovaries were collected at a local abattoir and transported to the laboratory within 3 h in sterilized saline containing 100 IU/ml gentamycin at 30 to35°C. Cumulus-oocyte complexes (COCs) were obtained by slicing the ovarian surface with a razor blade. COCs with more than three cumulus layers and a finely granulated homogeneous ooplasm were selected and cultured in TCM 199 medium supplemented with 10% FBS, 10 µg/ml FSH, 10 µg/ml LH, 1 µg/ml E_2 and 100 IU/ml penicillin/streptomycin for 19 to 21 h at 38.5°C under 5% CO_2 in humidified air. Then cumulus cells were removed from the oocytes by manual pipetting in the presence of 1

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Abbreviation: NT, Nuclear transfer; **hlF,** human lactoferrin; **KSOM,** potassium simplex optimization medium; **TCM,** tissue culture medium; **BSA,** bovine serum albumin.

Table 1. Effect of culture medium on the embryo development competence.

Treatment	Number of embryo cultured	Number (%) of embryo developed to	
		Cleaved (2 day)	Blastocyst (7 day)
TCM 199	168	124 (73.6 ± 4.2)[a]	18 (10.6 ± 2.1)[a]
KSOM -TCM 199	122	91 (74.0 ± 3.9)[a]	24 (19.5 ± 1.4)[b]

Values in the same column with different superscripts are significantly different ($P < 0.05$).

mg/ml of hyaluronidase, and oocytes with extruded first polar body were selected for enucleation.

Preparation of recipient oocytes

Denuded oocytes with a polar body were incubated in TCM 199 supplemented with 7.5 µg/ml cytochalasin B, 10 µg/ml Hoechst 33342 and 10% FBS at 38.5°C for 10 min. First, the polar body was placed at the 2 o'clock position, and the location of the chromosomes was determined after a brief exposure of the cytoplasm to UV light, then the polar body and nucleus were removed by aspiration using a pipette placed at the 3 o'clock position.

Nuclear transfer

A cell was slipped into the perivitelline space of the enucleated oocyte. Nuclear transfer couplets were electrically fused in medium comprising of 0.25 M D-Sorbitol, 0.5 mM $(CH_3COO)_2$ $Mg.4H_2O$, 0.1 mM $(CH3COO)_2$ Ca, 0.5 mM Hepes and 1 mg/ml bovine serum albumin (BSA), by applying a single electric pulse (20 µs each, 1.2 kv /cm). Activation of fused embryos was achieved chemically by incubation in 5 µM ionomycin for 5 min at room temperature, and were then incubated at 38.5°C under 5% CO_2 in humidified air in TCM 199 containing 2 mM 6-DMAP for 4 h.

Embryo culture and transfer

After activation treatments, the reconstructed embryos were transferred to equilibrated KSOM media (Millipore, USA), and cultured undisturbed for 36 to 40 h at 38.5°C under 5% CO_2 in humidified air. The NT embryos that had been cultured for 40 to 44 h after fusion were transferred into oviducts of the recipients at 40 to 48 h after estrus, respectively.

PCR analysis

The goat ear cells, donor cells and surrogate skin cells were resuspended in lysis buffer (40 mM Tris, pH 8.9, 0.9% TritonX-100, 0.9% NonidetP-40 and 0.4mg/mL proteinase K), incubated at 55°C for 5 h, and heated to 95°C for 10 min in order to inactivate proteinase K. The genomic DNA was amplified using a PCR machine (PE Applied Biosystems, Foster City, CA, USA) in a 20 µL reaction volume with the following variables: 30 cycles of 1 min at 95°C, 1 min at 62°C and 1min at 72°C and a final 10 min extension period. The PCR primers set were used to amplify 750 bp *hLF* gene. The forward primer and reverse primer are as follows: 5′-GAATGGCTGGCAGTGAAACA-3′ and 5′-CTCAATGGGCTCAGGTGGAC-3′.

Experimental designs

Experiment 1: After activation treatments, the reconstructed embryos were cultured in groups of 10 in 35 µL drops of KSOM or TCM 199 under mineral oil in 5% CO_2 in humidified air. Reconstructed embryos were allocated to two culture medium treatments: (1) TCM 199 + 0.1% BSA for 2 days, then TCM 199 + 10% FBS until Day 7; (2) KSOM for 2 days, then TCM 199+10% FBS until day 7.

Experiment 2: We divided Day-2 (48 h after fusion) reconstructed embryos into four groups based on number of blastomeres: 2-cell; 3- to 4-cell; 5- to 8-cell; and >8-cell. They were cultured in the same conditions, and formation rates of blastocyst were counted after 7 days. Besides, each was stained with Hoechst 33342 to observe the situation of blastomere nuclei.

Statistical analysis

For each treatment, at least three replicates were run. Statistical analyses were carried out by ANOVA (multiple comparisons are made with Bonferroni test). Data are expressed as mean ± SE, and $P < 0.05$ is considered significant.

RESULTS

Effect of culture medium on the embryo development competence

The efficiency of TCM 199 and KSOM-TCM 199 medium on the transgenic embryos developmental capability were examined (Table 1). The cleavage rate in culture system of TCM 199 and KSOM-TCM 199 were 73.6 and 74%, respectively ($P > 0.05$). Meanwhile, the blastocyst rate in KSOM-TCM 199 medium was greater than that in TCM199 medium (19.5 and 10.6%, respectively, $P < 0.05$), thus indicating that the KSOM-TCM 199 culture system might be more suitable in culturing transgenic NT embryos *in vitro*.

The formation rates of blastocysts from *hLF* transgenic cloned embryos (48 h after fusion)

The cleavage of *in vitro* cultured *hLF* transgenic cloned embryos was not fully synchronized, and their development capacity was also different (Table 2). The blastocyst formation rate of NT embryos, which were at 3-4 cell (31.9%) and 5-8 cell (28.2%) stage 48 h after fusion were greater than those at 2-cell (9.1%) and > 8-cell (8.3%) stages ($P < 0.05$). This result may indicate that the number of Day-2 embryos at 3-8 cell stage (79.46%) was more than those at 2-cell (8.90%) and > 8-

Table 2. The formation rate of blastocysts from *hLF* transgenic cloned embryos (48 h after fusion).

Stage of embryos at day 2	Number of embryo	Proportion of stage of embryo at day 2 (%)	Number (%) of blastocysts at day 7
2-cell	26	8.90	3 (9.1±4.1)[a]
3-4 cell	91	31.17	28 (31.9±2.7)[b]
5-8 cell	141	48.29	38 (28.2±2.8)[b]
>8-cell	34	11.64	4 (8.3±3.9)[a]

Values in the same column with different superscripts (a and b) are significantly different ($P < 0.05$).

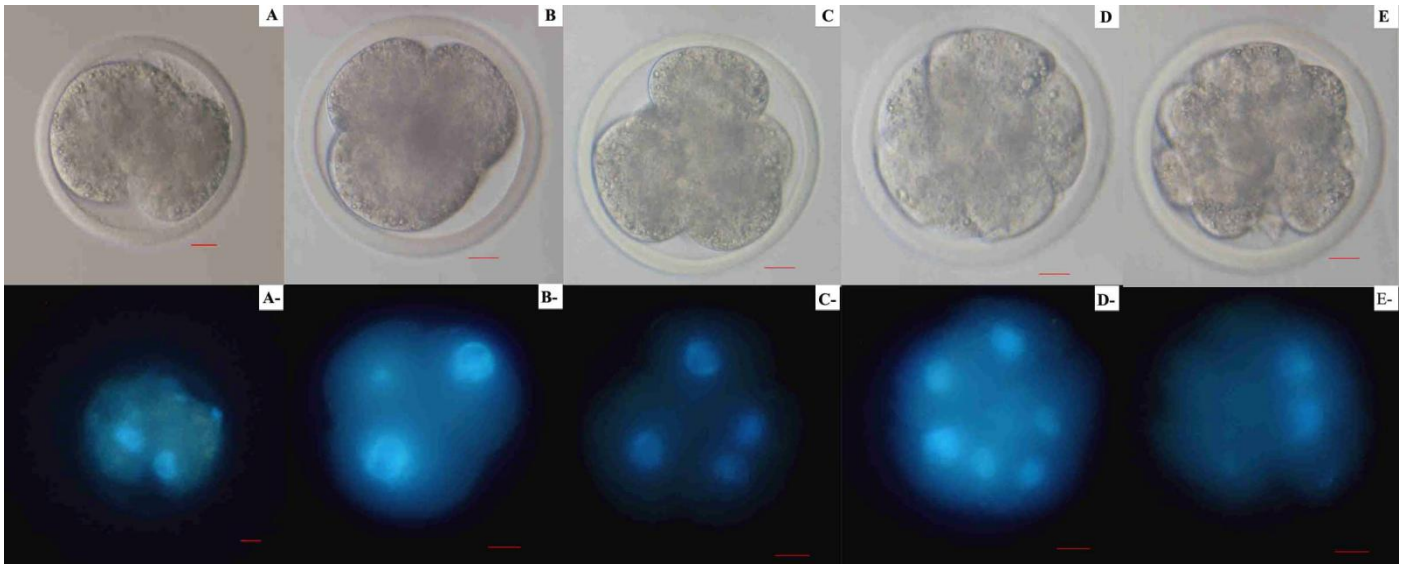

Figure 2. Transgenic cloned embryos developed *in vitro* (48 h after fusion). A, B, C, D, E are 2-cell, 3-cell, 4-cell, 8-cell and >8-cell under bright view, A-, B-, C-, D-, E-, are 2-cell, 3-cell, 4-cell, 8-cell and >8cell under UV. Scale bar = 20 µM.

cell stages (11.64%), whose blastocyst development capacity was negatively affected by some factors.

We could find that the number of Day-2 embryo cell nucleus at 2-cell stage (Figure 2A-) and 3-8 cell stage (3-cell Figure 2B-, 4-cell 2C- and 8-cell 2D-) was 2, 3, 4 and 7, respectively by Hoechst 33342 staining in accordance with the number of their blastomeres (Figure 2A to D). However, the Day-2 embryos at > 8-cell stage (Figure 2E-) mostly reflected a high frequency of fragmentation (> 50%), which appeared blastomere-like without nucleus existed.

Nuclear transfer to produce transgenic goat

The NT embryos were cultured in KSOM medium for 2 days and surgically transferred to surrogate goats. In total, we had 536 reconstructed embryos before activation with Ionomycin and 6-DMAP, and 371 (69%) embryos cleavaged *in vitro* culture in the present study. The Day-2 embryos at 2-cell and > 8-cell stages were abandoned and a total of 304 (82%) SCNT embryos were selected and transferred into 43 recipient females (Table 3). Finally, 9 pregnancies were detected by non-return estrus and B ultrasound scanner, while 5 healthy female lambs was delivered (Figure 3A to C). However, 2 NT dairy goats had perinatal death, 1 fetus was aborted at Day 118 (Figure 3D), and 3 of the pregnancies failed to maintain to term. The presence of the *hLF* gene in the 5 lambs was confirmed by PCR using DNA from ear cells of each lamb (Figure 4).

DISCUSSION

The combination of somatic cell nuclear transfer (SCNT) and transgenic technology led to the production of transgenic cloned animals. During the process, the *in vitro* culture system was a pivotal step. Although, there were a variety of mediums such as TCM 199 (Baguisi et al., 1999), Charles Rosenkrans medium **(**CR1) (Lan et al., 2006), and synthetic oviductal fluid (SOF) (Melican et

Table 3. Results of *hLF* transgenic cloned offspring by using KSOM medium

Number fused Couplets	Number (%) embryos (cleavage stage)	Number (%) of embryo transferred	Transferred recipient	Pregnant recipient	Abortive recipient	Viable offspring
536	371 (69)	304 (82)	43	9	4	5

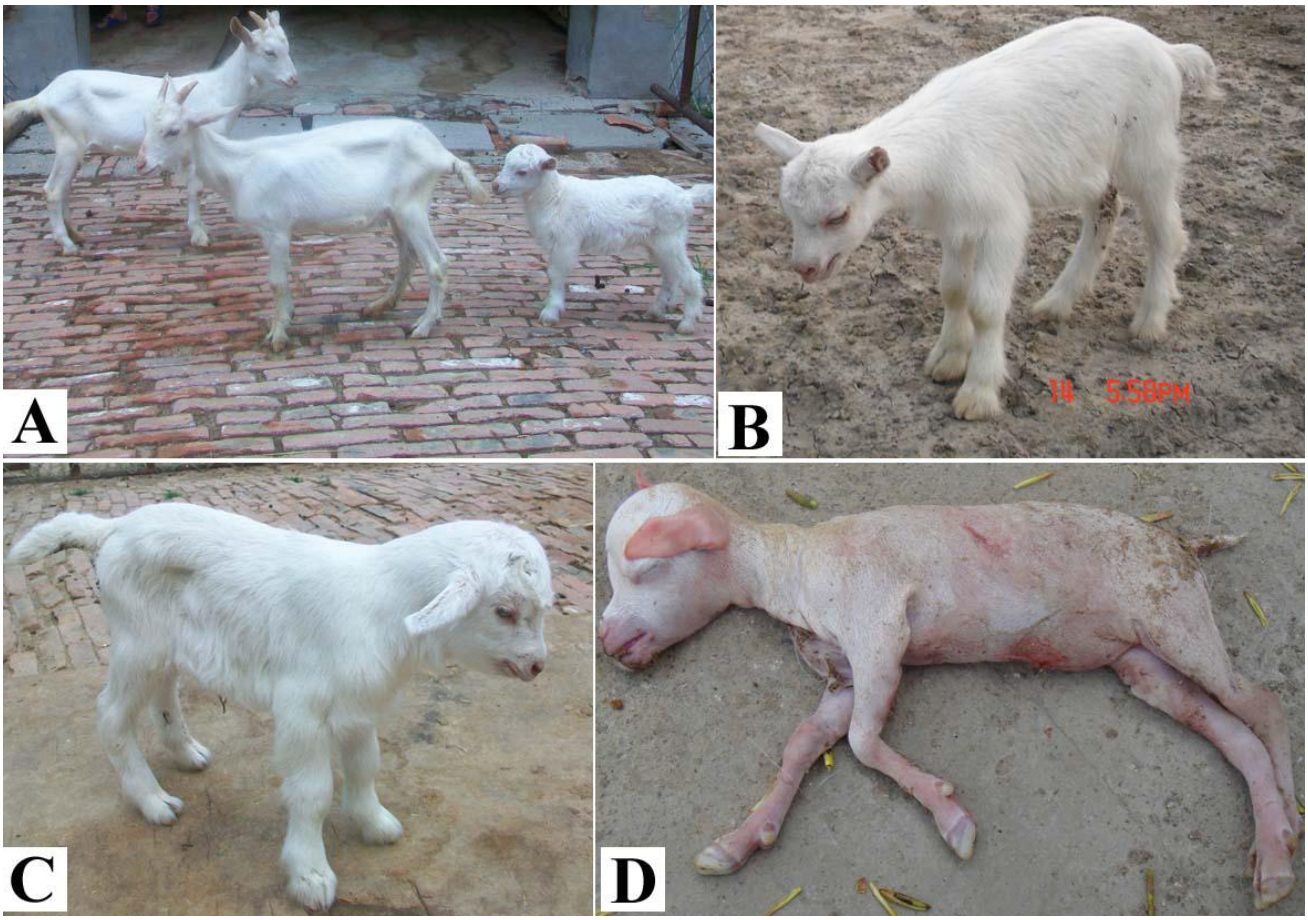

Figure 3. Cloned kids derived from the *hLF* gene transgenic fibroblast cells. (A) Three survived NT dairy goats; (B, C) two NT dairy goats that died perinatally; (D) aborted fetus at Day 118.

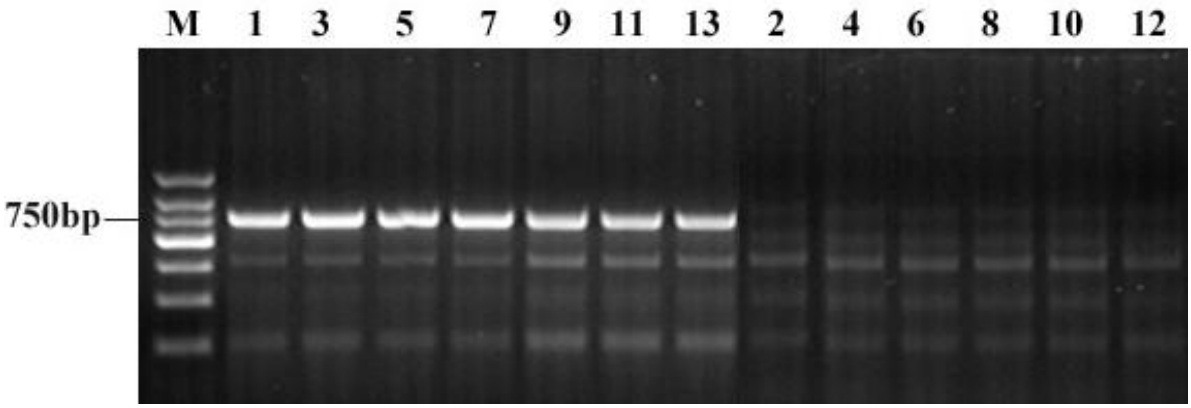

Figure 4. Identification of *hLF* gene in the cloned kid by PCR amplification. Lane 1. Donor cell; lanes 3, 5, 7, 9 and 11, the cloned kid; lane 13, aborted fetus; lanes 2, 4, 6, 8, 10 and 12, the recipient goat as negative control.

al., 2005) shown to culture the early goat embryos *in vitro*, none transgenic offspring was produced by KSOM medium. In the present study, we successfully produced *hLF* transgenic cloned dairy goat and confirmed the KSOM medium as an ideal culture medium for transgenic cloned embryos during the cleavage stage. *In vitro* culture medium, KSOM was superior to TCM 199 in culturing the transgenic cloned embryo at cleavage stage, and the embryos cultured by the KSOM were transferred into the synchronized recipient goats. At last, 4 healthy transgenic cloned dairy goats were produced.

The metabolism of embryos cultured *in vitro* was maintained by energy substances such as glucose, lactate and pyruvate. Glucose had been shown to be very important in the process of blastocyst formation. Nevertheless, high concentration of glucose would block the development of embryo during the cleavage stage (Schini and Bavister, 1988; Chatot et al., 1989; Lawitts and Biggers, 1991; Thompson et al., 1991). These contradictory results were explained by a biphasic effect of glucose: lower concentrations (< 3 mM) stimulated early cleavage stage embryo development, whereas high concentrations (3 - 5 mM) inhibited early embryo development (Lim et al., 2007). Our study also confirmed that high concentrations inhibited early embryo development. In the present study, the glucose concentration in KSOM medium was 0.2 mM, whereas the glucose concentration in TCM 199 medium was 5.6 mM. Therefore, our result demonstrated that the KSOM medium was more appropriate than TCM 199 medium to culture the SCNT embryos from the pronuclear to the cleavage stage.

Currently, most of early goat NT embryos at the cleavage stage (48 h after fusion) were used for embryo transfer (ET) (Baguisi et al., 1999; Reggio et al., 2001; Melican et al., 2005; Chen et al., 2007). It was essential to screen the NT embryos quality before ET. Since *in vitro* culture embryos were influenced by several factors, the development of pre-implantation embryo was not synchronized. In the Day-2 embryos in the present study, the blastocyst formation rate of 3-8 cell stage embryos was greater than that of other stage embryos. Furthermore, similar studies have also shown that 3-8 cell stage embryos had lower chromosome abnormalities (Ulloa Ullo et al., 2008a, b). Therefore, it is reasonable to believe that chromosome abnormality may be related to the development capability of cleavage-stage embryos, which lead to a lower blastocyst formation rate (Magli et al., 2007; Ulloa Ullo et al., 2008b). In addition, we also found that most transgenic cloned embryos were in 3-8 cell stage embryos at Day 2 (79.46%). Meanwhile the > 8-cell stage embryos showed higher frequency of fragmentation (> 50%), which might be the debris due to the abnormal embryonic development, and this might also be the reason of the low formation rate of blastocyst. Therefore, it was necessary to eliminate transgenic cloned embryos with abnormal morphology and select the 3-8 cell stage embryos before ET.

In conclusion, KSOM could be selected as the early embryo culture medium. In order to enhance the efficiency of transgenic cloned dairy goat production and decrease the economic cost, selecting the 3-8 cell stage transgenic cloned embryos (48 h after fusion) with good morphology was necessary when the embryos were transferred to synchronized recipients.

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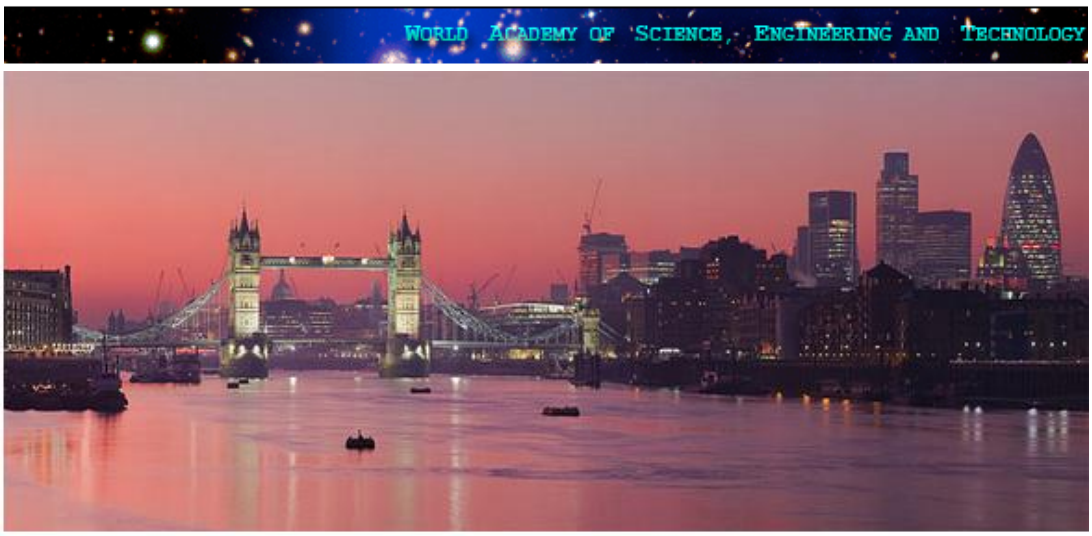

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