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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

Quantitative PCR analysis of diesel degrading genes of *Acinetobacter calcoaceticus* isolates

Johnson Lin*, Vikas Sharma and RakshaToolsi

School of Life Sciences, University of KwaZulu-Natal (Westville), Private Bag X 54001, Durban, Republic of South Africa.

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Acinetobacter **strains LT_1 and V_2 were grown in Bushnell-Haas medium with 1% diesel and their expression levels of eight diesel-degrading genes were evaluated by quantitative polymerase chain reaction (PCR). LT_1 and V_2 isolates achieved 86.2 and 89.7% degradation respectively, after 60 days with no significant differences in expression, in comparison with 16S rRNA, *rub*B and *lip*B. LT_1 showed higher expression levels of *rub*A, *alk*M, *alk*R and *xcp*R genes during the initial stages of incubation corresponding to higher level of degradation rate. Amplification of *alk*M, *alk*R and *xcp*R genes of V_2 indicated more than one enzyme system involved in the process. Low *lip*B and *lip*A expression in LT_1 and no *lip*A expression in V_2 suggested the absence of lipases in degradation; however, *est*B gene was predominantly expressed in V_2. Thus, isolates LT_1 and V_2 possessed comparable efficiency in degrading diesel; however, more complex systems have been employed by V_2i n degradation process.**

Key words: qPCR, diesel degradation, *Acinetobacter calcoaceticus,* bioemulsifier, gene expression.

INTRODUCTION

A wide array of studies has dealt with biodegradation and bioremediation of petroleum hydrocarbon unraveling the intricacies of degradation and generating a wealth of bacterial, genetic, biochemical, and physiological knowledge (van Beilen and Funhoff, 2007; Rojo, 2009). Characterization of bacterial communities, isolation of prospective degraders, monitoring their response to pollutants, and identifying functional genes and enzymes involved in biodegradation processes have been subject to extensive research (Wentzel et al., 2007; Rojo, 2010). Over the years, a multitude of bacterial species capable of degrading hydrocarbon, have been readily isolated from hydrocarbon-contaminated and non-contaminated sites. *Acinetobacter* sp. are one of the representative genera capable of utilizing *n*-alkanes (Tani et al., 2002; Van Hamme et al., 2003; Singh and Lin, 2008; Lee et al., 2012; Mara et al., 2012).

The pathways of alkane degradation and the enzymes involved in it have been reviewed extensively (Coon, 2005; van Beilen and Funhoff, 2007; Wentzel et al., 2007; Rojo 2010). In majority of the cases, aerobic degradation of n-alkanes starts either by the terminal (van Beilen and Funhoff, 2007; Wentzel et al., 2007; Rojo 2010) or sub-terminal oxidation pathway (Kotani et al., 2006). In this pathway, conversion of methyl group which leaves a primary alcohol, is further oxidized to the corresponding aldehyde, fatty acid and finally processed by β–oxidation pathway to generate acetyl-CoA (Wentzel et al., 2007; Rojo 2010). In some microorganisms, both terminal and sub-terminal oxidation pathway coexists (Watkinson and Morgan, 1990).

In *Acinetobacter calcoaceticus*, alkane hydroxylases

*Corresponding author. E-mail: linj@ukzn.ac.za. Tel: +27-31-2607407. Fax: +27-31-2607809.

(encoded *alk*M) require two soluble electron transfer proteins named rubredoxin (*rub*A) and rubredoxinreductase (*rub*B) for *n*-alkane degradation and are obligatory for its function (Tani et al., 2001). The *alk*M gene expression is regulated by the alkane hydroxylase regulator (*alk*R) (Ratajczak et al., 1998). The participation of *xcpR* gene in alkane degradation was proposed in *A. calcoaceticus* ADP1 after the insertional inactivation of *xcpR* impeded the secretion of lipase and esterase that lead to the lack of growth on dodecane and slower growth on hexadecane (Parche et al., 1997). In *A. calcoaceticus* ADP1, the *est*B gene encodes a functional esterase and is localized in an operon with genes *rub*A, *rub*B and *oxy*R (Geißdörfer et al. 1999). The esterase activity has been reported to be involved in the sub-terminal oxidation pathway (Kotani et al., 2007). The involvement of these enzymes in the degradation of hydrocarbons has been proposed, however their exact function is unknown.

Genomes of many *Acinetobacter* strains have been sequenced recently, including those of *Acinetobacter* sp. DR1 (Jung et al., 2010), *Acinetobacter oleivoran s*sp. DR1(T) (Kang et al., 2011), *Acinetobacter venetianus*RAG-1T (Fondi et al., 2012) and *Acinetobacter venetianus*VE-C3 (Fondi et al., 2013). Although, momentous gains have been made in understanding the processes in hydrocarbon degradation, the minutiae of individual systems as well as the diversity of systems have yet to be fully articulated (Van Hamme et al., 2003). Therefore, this study investigated the proficiency of *A. calcoaceticus* isolates in the degradation of diesel and implementing the levels of mRNA expression of diesel degrading genes using quantitative polymerase chain reaction.

MATERIALS AND METHODS

Selection and identification of diesel degrading bacterial isolates

A. calcoaceticus isolates $LT_1$and V_2, were obtained from departmental stock cultures. LT_1 (accession number JN036553) were originally isolated from diesel-contaminated soil (Singh and Lin, 2008) while V_2 (accession number JN036552) was isolated from used engine oil-contaminated soil (Mandri and Lin, 2007) previously from the same laboratory Isolates were streaked on nutrient agar and incubated at 30°C for 24 h to attain pure cultures.

Growth of bacterial isolates on diesel as sole carbon and energy source

To access their ability to degrade diesel, isolates were grown on Bushnell-Haas (BH) minimal medium with diesel as sole carbon source (Singh and Lin, 2008). Isolates were grown in separate 100 ml Erlenmeyer flasks with a total volume of 25 ml of liquid BH medium supplemented with 1% (vol/vol) sterilized diesel using 0.22 µm membrane filters (Whatman) as a carbon and energy source. The isolates were grown overnight at 30°C in Luria broth, washed thrice with normal saline and 1% of the washed cells were inoculated in each BH medium (standardized using BH medium to OD_{600} of 1.0 after centrifugation). The BH medium flasks were incubated aerobically at 30°C in a rotary shaker (160 rpm) for variable time period. An abiotic control flask, with 1% (vol/vol) diesel and devoid of bacterial inoculum, was incubated under the same conditions to ascertain abiotic loss. Bacterial growth was monitored by optical density at 600 nm (OD_{600}) at 5, 10, 15, 20, 30, 40, 50 and 60 days of incubation. The experiments were repeated in triplicate.

Carbon source utilisation patterns were determined by gravimetric analysis (Marquez-Rocha et al., 2001). The remaining diesel in each sample was extracted using the separating funnel with 10 ml dichloromethane three times. The dichloromethane extract was funneled through filter paper containing 5 g anhydrous sodium sulfate (Na_2SO_4) for the removal of cellular debris and absorption of moisture. The extract was collected in the pre-weighed glass tubes and was left overnight in a fume cupboard to facilitate evaporation of dichloromethane. The combined mass of the pre-weighed tube and diesel was recorded. The mass of the tube was subtracted to determine the mass of the diesel remaining. The mass of the diesel remaining was subtracted from the mass of diesel in the control sample and multiplied by 100 to determine the percentage of diesel remaining. Student t-test (Microsoft Excel 2010) was used to evaluate the differences of diesel degradation between LT_1 and V_2. Probability (significant level) was set at 0.05.

RNA extraction and cDNA validation

The BH medium containing isolates was centrifuged at 4000 x*g* for 20 min resulting in a pellet and a cell-free supernatant. Pellet was re-suspended in phosphate-buffered saline (PBS), pH 7.6, and subjected to further centrifugation at 4000 x*g* for 20 min. Total RNA was extracted from each pellet using Aurum ^{TM} Total RNA Mini Kit (BIO-RAD) according to manufacturer's instructions with following modifications :500 µl of lysozyme was added instead of 100 µl of 1000 µg/ml stock dissolved in TE (10 mMTris, 1mM EDTA, pH 7.5) to the bacterial suspension and incubated at room temperature for 30 min. Total RNA purity and yield was determined spectrophotometrically using Nanodrop ND-1000 (BIO-RAD). Purified RNA (1 µg) was used to prepare cDNA by using a first Strand iScript^{TM} cDNA Synthesis Kit (BIO-RAD). The reverse transcriptase PCR was performed (GeneAmp® PCR System 9700, Applied Biosystems) according to manufacturer's instructions. Purified RNA and cDNAwas also assessed electrophoretically by ethidium bromide staining on 2% (wt/vol) agarose in 1 X TAE running buffer at 80V. The $OD_{260/280}$ ratio obtained ranged from 1.30 to 2.27. Samples LT_1 - day 5, with ratio of 1.30, indicated poor RNA quality that resulted in no 16S rRNAPCR product detected. LT_1 - day 5 sample was omitted from the real time PCR study.The PCR product of 16S rRNA gene using 63F and 1387R primers (Marchesi et al., 1998) was obtained. DNA concentrations of 16S rRNA PCR products were measured spectrophotometrically using a NanoDrop ND-1000 spectrophotometer and concentrated stock solutions of specific 16S rRNA gene fragments were prepared. These were serially diluted to generate a set of calibration pure standards with known concentrations of target DNA.The cDNA obtained was stored at -20°C.

Real-time PCR data analysis and quantification of gene expression

The nucleotide sequences of the genes of interest were acquired from the National Center for Biotechnology Information (NCBI) nucleotide sequence databases with the following accession numbers: AF047691 (*lip*A and *lip*B), AJ233398 (*alk*M and *alk*R), EF524340 (16S rRNA), Y09102 (*xcp*R) and Z46863 (*rub*A, *rub*B,

Table 1. Primers sequences for real-time PCR amplification of target genes.

Primer	Sequence	Product size (bp)
16S rRNA		
Forward	5'-GTAGCGGGTCTGAGAGGATG-3'	169
Reverse	5'-GCCTCCTCCTCGCTTAAAGT-3'	
*rub*A (rubredoxin)		
Forward	5'-GATTTATGATGAAGCCGAAGG-3'	91
Reverse	5'-GTCAGGGCAAGTCCAGTCAT-3'	
*rub*B (rubredoxin reductase)		
Forward	5'-GCCCACTGGGTCGTCTATTA-3'	222
Reverse	5'-CGTGTTTTGCCAGATCAATG-3'	
*alk*M (alkane hydroxylase)		
Forward	5'-AAAGATGCGCGTAATCCAAC-3'	189
Reverse	5'-ATTAATGGCACCCATCGAAA-3'	
*alk*R (alkane hydroxylase regulator)		
Forward	5'-TGTAGCATGATGCGCTTTTC- 3'	161
Reverse	5'-CACAAGGTGAATGGGCTTTT- 3'	
*est*B (esterase)		
Forward	5'-ATCCAAAATTCGCCACAAAG-3'	150
Reverse	5'-TTTTTAATCCGCATCGCTTC-3'	
*Lip*A (lipase)		
Forward	5'-CTTCCGTTTCAACGATTGGT- 3'	189
Reverse	5'-TATACGCTGCACCGACAGAG- 3'	
*lip*B (lipase)		
Forward	5'-CCAACCCTAGCAGCATCATT- 3'	153
Reverse	5'-TGCAACAAGCTCTGCTTCAG- 3'	
*xcp*R (a subunit of the general secretion pathway for exoproteins)		
Forward	5'-AGGGTTAATGGCGGAAGACT-3'	192
Reverse	5'-CCAATCCCTTCGAGCTGATA-3'	

and *est*B). Primers for real-time PCR were designed with the aid of primer design software Primer3 (Kubista et al., 2006) and were synthesized by Inqaba Biotech. The real-time PCR primer sequences are presented in Table 1.

PCR amplifications of *alk*M, *alk*R, *rub*A, *rub*B, *est*B, *lip*A, *lip*B, *xcp*R and 16S rRNA genes were performed using LightCycler® Instrument Version 3.5real-time PCR system (RocheDiagnostics). LightCycler® reactions were performed in 20 µl glass capillary tubes using LightCycler® FaststartDNA Master SYBR Green I kit (Roche Diagnostics) according to the manufacturer's instructions. The evaluating parameters selected for data analysis were fluorescence (d[F1]/dT), melting temperature (T_m) and crossing point (C_p) (Pfaffl et al., 2002; Rasmussen, 2001). Conditions of real-time PCR for the respective target genes under the optimized conditions are presented in Table 2. Specificity of real-time PCR primers was determined using LightCycler Software®, Version 3.5 (Roche Diagnostics). Due to the multiple products in some samples, the cDNA of $LT_1$was selected as the calibrator for 16S rRNA, *alk*M, *alk*R, *rub*A, *lip*A, *lip*B and *xcp*R genes and that of $V_2$for *est*B gene. Each amplification product for the target genes demonstrated a specific and characteristic melting curve (supplementary Figure 1S). Specificity of real-time PCR products were documented by agarose gel electrophoresis and resulted in a single product of anticipated length. No PCR amplification product was observed for gene *rub*B, despite rigorous optimization strategies and redesigning of primers.

Equal aliquots of cDNA obtained were standardized to 1000 ng/µl.

To generate a standard curve, the serially diluted cDNA standard (1,000 to 0.001 ng) was quantified in each real-time PCR runin duplicates. The efficiency of each standard curve was determined using the equation: Efficiency (E) = $10^{-1/slope}$ - 1 (Rasmussen, 2001). Descriptive statistics (minimal (Min) and maximal (Max) mean values, standard deviation (SD), and coefficient of variance (CV %), of the derived C_p values were computed for each investigated gene to determine intra-sample variation.

Gene expression was quantified using the Pfaffl model which combines gene quantification and normalizationand was calculated with the aid of Microsoft Excel® based application, Relative Expression Software Tool - XL (REST-XL©) - Version 2 (Pfaffl et al., 2002). The C_p values of both, control and the samples of interest were normalized based on the PCR product of 16S rRNA gene in each sample. The copy number of each gene was calculated based on the 16S rRNA standard curve.

RESULTS AND DISCUSSION

Growth of bacterial isolates on diesel and their carbon utilization pattern

The growth behavioral patterns of the bacterial isolates on diesel were monitored over a period of 60 days and determined by optical density (OD_{600}). The results obtained

Table 2. Composition of reaction Mastermix and amplification parameters for LightCycler®real-time PCR assays.

Target gene	Primer conc. (pmol/µl)	Reaction components (µl)*					Amplification parameter
		DEPC H_2O	Forward primer	Reverse primer	$MgCl_2$ (25 mM)	SYBR green	
16S rRNA	1.25	5.8	0.5	0.5	1.2	1.0	30 cycles of 5 s at 95°C (Denaturing), 10 s at 62°C (Annealing), 5 s at 72°C (Polymerizing)
*alk*M	10.0	6.0	0.5	0.5	1.0	1.0	50 cycles of 5 s at 95°C, 15 s at 62°C, 5 s at 72°C
*alk*R	5.00	6.2	0.5	0.5	0.8	1.0	55 cycles of 5 s at 95°C, 8 s at 62°C, 5 s at 72°C
*rub*A	5.00	5.8	0.5	0.5	1.2	1.0	40 cycles of 5 s at 95°C, 15 s at 62°C, 5 s at 72°C
*lip*A	10.0	5.8	0.5	0.5	1.2	1.0	55 cycles of 5 s at 95°C, 10 s at 62°C, 5 s at 72°C
*lip*B	10.0	5.8	0.5	0.5	1.2	1.0	55 cycles of 5 s at 95°C, 10 s at 62°C, 5 s at 72°C
*xcp*R	10.0	5.8	0.5	0.5	1.2	1.0	55 cycles of 5 s at 95°C, 10 s at 62°C, 5 s at 72°C
*est*B	10.0	5.8	0.5	0.5	1.2	1.0	55 cycles of 5 s at 95°C, 15 s at 60°C, 5 s at 72°C

*Final reaction volume of 9 µl.

obtained are presented in Figure 1. After 60 days of incubation, a 9.40% mass reduction in the diesel of the abiotic control flask was detected (data not shown). Both isolates displayed comparable proficiency in the overall utilization of diesel as a sole carbon and energy source. Rapid degradation of alkanes occurred during the first 5 days of incubation with attributes to the exponential growth phase of the bacterial cells. The $OD_{60\ 0}$increased progressively up to 15 days, and remained relatively constant until the end of the culture time. Both isolates LT_1 and V_2 showed diesel degrading capability. LT_1 and V_2 achieved 58.6 and 48.3% degradation after 5 days of incubation and 86.2and 89.7% degradation after 60 days of incubation, respectively (Figure 1). During the initial days of incubation, V_2 degraded less diesel as compared to LT_1. However, after a rapid increase in diesel degradation at day 15, V_2 emerged as the most proficient diesel degrader by the end of the incubation period (Figure 1), though the diesel degradation ability between these two strains did not differ significantly (p=0.506) at the end of the experiment.

The presence of an opaque substance was observed in the hydrocarbon and hydrocarbon - culture medium fraction of V_2 samples throughout the incubation process. After 10 days of incubation, however, the diesel of the V_2 samples no longer appeared as a confluent layer over the culture medium phase, but rather as minuscule droplets. This occurrence was not observed for LT_1 samples for the duration of the culture time.

Optimizing the components of the real-time PCR master mix

LightCycler analyses of *alk*M, *alk*R, *rub*A, *rub*B, *xcp*R, *est*B, *lip*A, *lip*B and16S rRNA were performed and optimized. Melting temperature (T_m) of target gene amplification products ranged from 79.3°C for *alk*R to 91.2°C for 16 rRNA (Figure 1S). A high degree of efficiency ranging from 1.76 to 2.24 was achieved indicating a stable and reliable assay. All generated standard curves illustrated high linearity with r^2 values of 1.00 over three orders of magnitude. An expression profile of *lip*A could not be created for LT_1, and V_2 samples due to very low expression levels of *lip*A and therefore, E_{lipA} could not be deter-

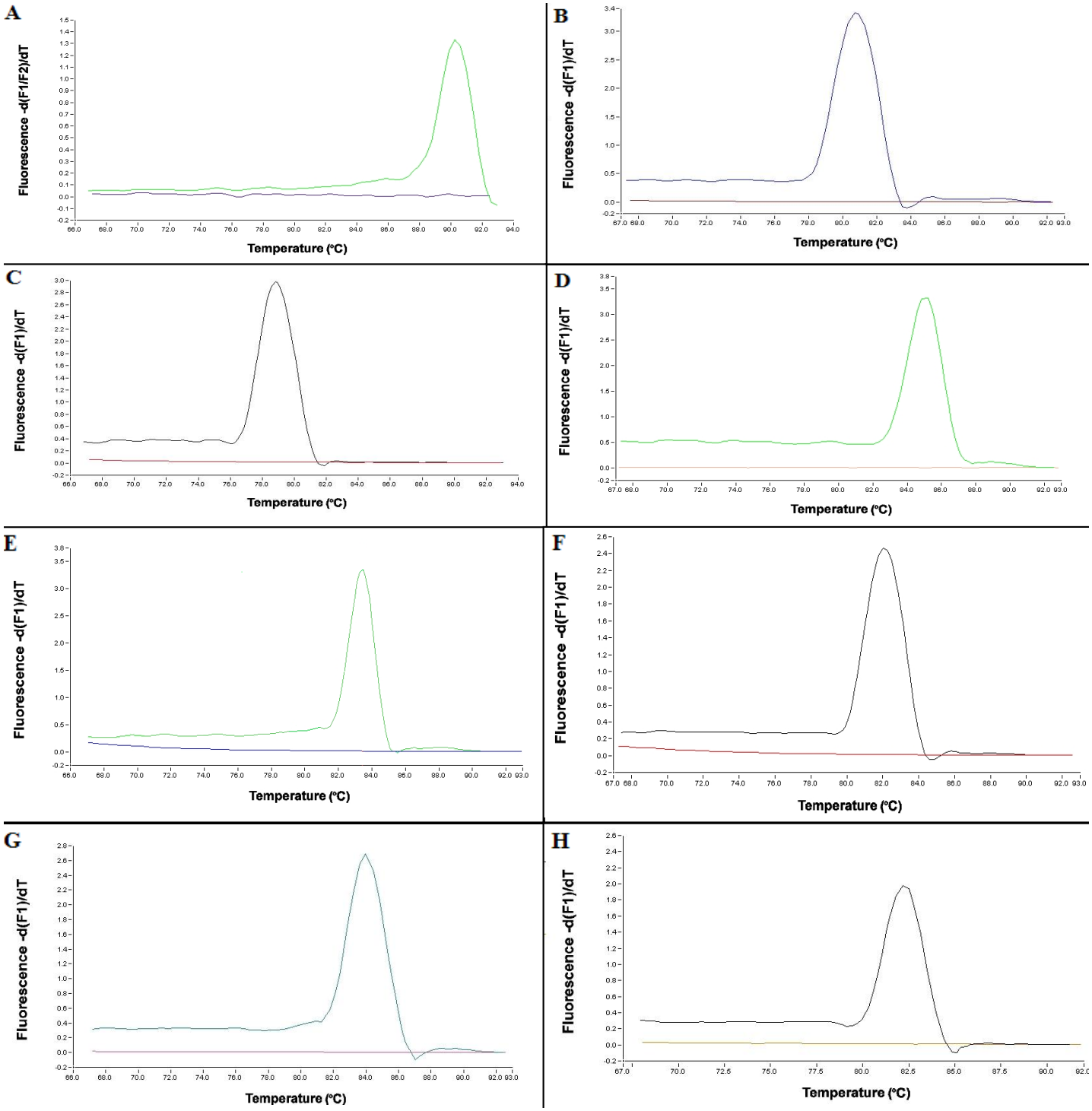


Figure 1S. Specificity of LightCycler® PCR. Amplification of target genes (A) 16S rRNA, (B) *alk*M, (C) *alk*R, (D) *rub*A, (E) *lip*A, (F) *lip*B, (G) *xcp*R, and (H) *est*B, as determined by melting curve analysis. Melting peaks were determined by plotting the continuous negative derivative of fluorescence emitted by each sample as PCR products were melted. The cDNA template control sample for each reaction does not show fluorescence, confirming the absence of primer-dimers.

mined. E_{alkR} yielded an efficiency of 2.24, exceeding the maximum permissible efficiency of 2 (E = 100%), indicating an inappropriately optimized assay and/or poor specificity of primer pairs.

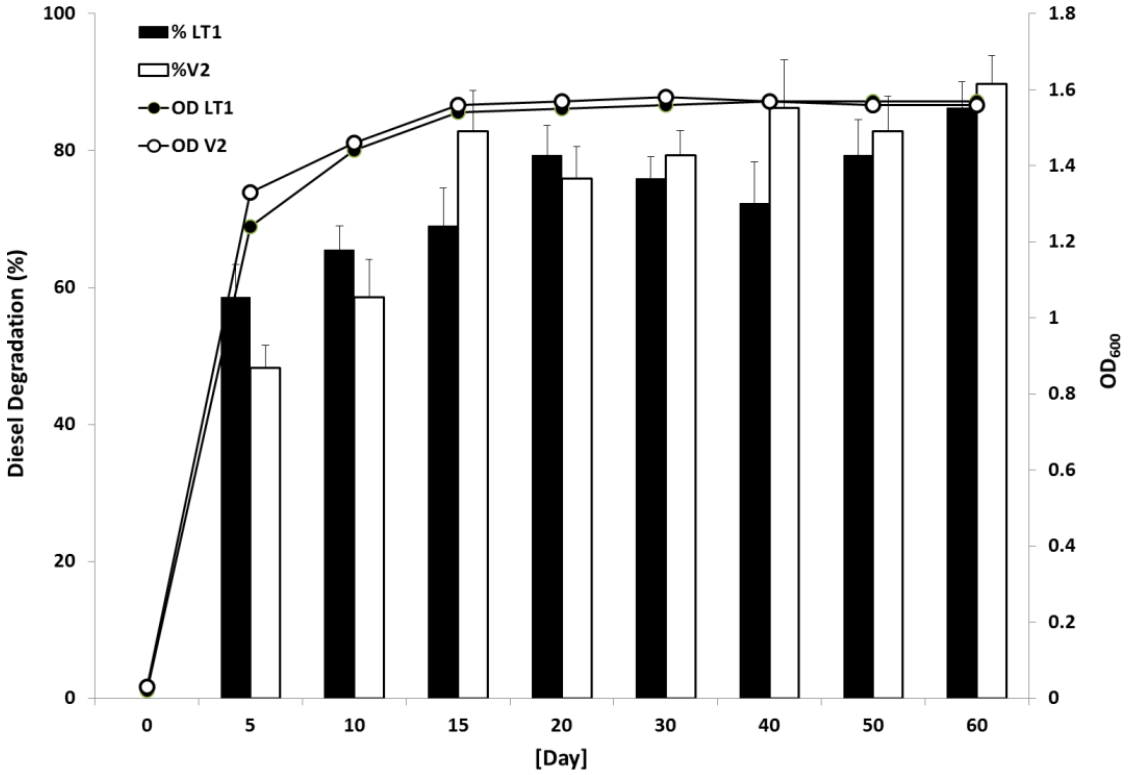


Figure 1. Diesel degradation (%) and growth (OD_{600}) patterns of *A. calcoaceticus* isolates.

The 16S rRNA gene was stably expressed in LT_1 samples throughout the time course and showed the lowest degree of intra-sample variation based on SD and CV% of C_p. The 16S rRNA gene of V_2 was not signi-ficantly different from 16S rRNA of LT_1 with *p* values of 0.072 demonstrating an accepted level of gene expres-sion variability. The findings also show the similar growth and diesel degradation patterns of both isolates as shown in Figure 1. Hence, the expressions of 16S rRNA of both isolates were used to normalize all other target gene expressions respectively for comparison.

The target gene expressions and comparison between LT_1 and V_2 isolates

The expression levels of *rub*A, *alk*M, *alk*R, and *xcp*R genes in LT_1 samples exhibited their maximums during the initial stages of incubation (Figures 2 and 3a) and with high degrees of intra-sample variation in C_p with SD and CV% (Table 3). The higher expression (8.71×10^{12} gene copies) of *alk*M gene was observed at day 10 as compared to other genes involved and its expression declined as incubation time progressed. The above results revealed the involvement of these gene products of LT_1 in the diesel degradation. Previous studies showed that *Acinetobacter* sp. strain ADP1 requires at least five essential genes, namely *rub*A, *rub*B, *alk*M, *alk*R, and *xcp*R, for *n*-alkane utilisation (Ratajczak et al., 1998) confirming *rub*A, *rub*B, and *alk*M constitute a three component alkane hydroxyase system in *A. calcoaceticus* (Ratajczak et al., 1998; Geißdörfer et al., 1999; van Beilen et al., 2002). The overall *alk*R gene expression of LT_1, samples was comparatively low in relation to the alkane hydroxylase complex genes, *alk*M and *rub*A. This inference is in accordance with preceding studies that found *alk*R to be expressed at lower levels during alkane degradation despite its indispensability in the degradation process (Ratajczak et al., 1998). The *xcp*R gene encodes a type IV pilus-related system that may not only be required for the secretion of proteins but may represent a more universal transport system for a variety of macromolecules. It may also facilitate the alleviation of membrane stress incurred by bacterial cells during alkane degradation (Parche et al., 1997). All genes except *lip*B gene demonstrated a similar pattern. The *lipB* gene was expressed in LT_1 at all sampling times, however, low expression levels was observed as depicted by the high C_p values (Table 3). Unlike other target genes that were expressed in the early stage of diesel degradation, expression levels of *lipB* gene increased with incubation time (from 5.62×10^8 gene copies at day 10 to 2.82×10^9 gene copies at day 20) for LT_1 and

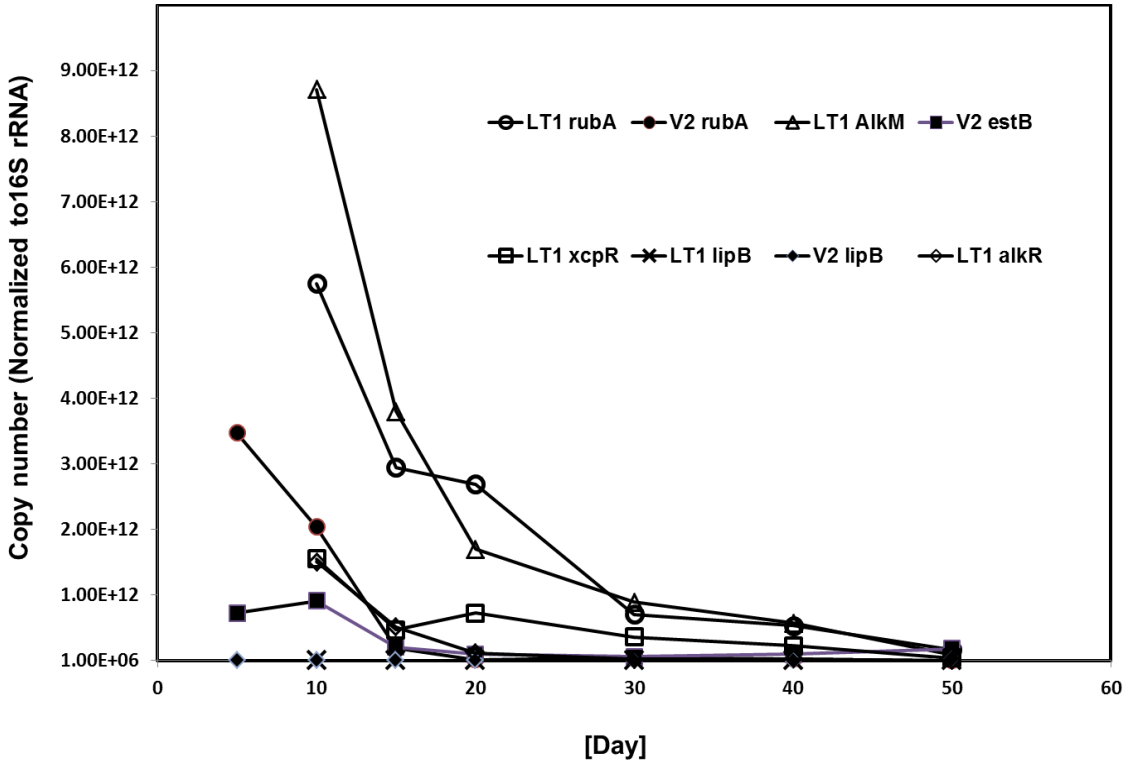


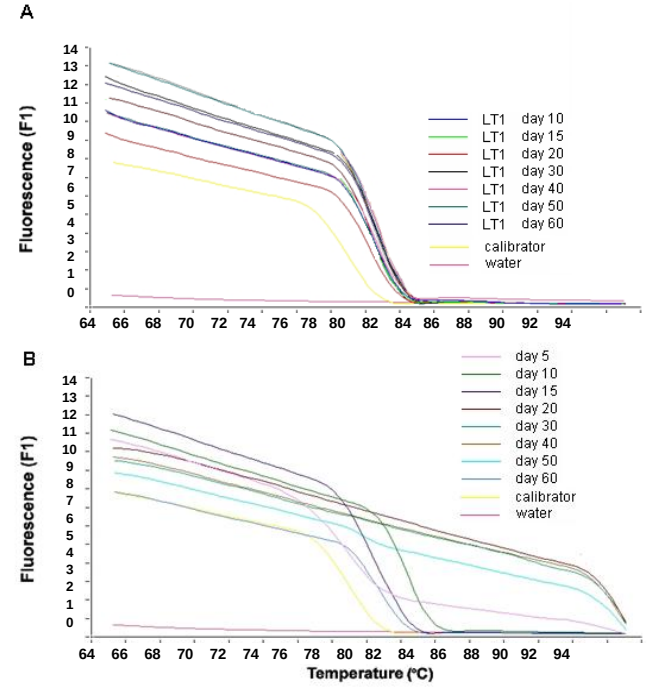
Figure 2. Expression profiles of *alk*M, *rub*A, *xcp*R, *lip*B and *alk*R genes of *A. calcoaceticus* LT_1 and of *rub*A, *est*B and*lip*B genes of *A. calcoaceticus* V_2.

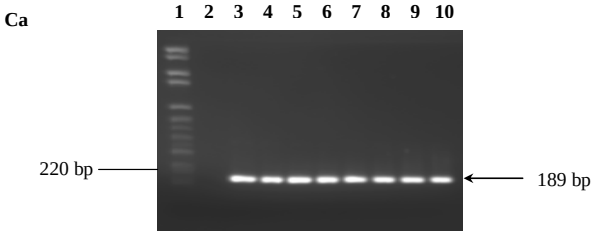
declined thereafter showing greater intra-sample variation (SD and CV% values of 10.27 and 25.10 respectively) (Table 3). Positive peak formations of desired *est*B and *lip*A gene products were observed for many samples. However, such peaks are a consequence of amplification occurring very late in the PCR often with C_p values > 51.00 in a 55 cycle amplification segment, therefore are considered invalid and are indicative of false-positive results. Lack of target-specific amplifications of *est*B and *lip*A in LT_1 samples, suggest either the absence of gene expressions or low levels of expression.

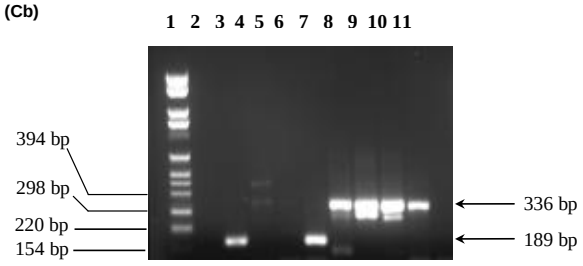
In comparison with the diesel-degrading target gene expression levels of LT_1, the expression profile of the *rub*A gene of V_2 had a similar trend as LT_1 (Figure 2), exhibiting the maximum during the initial stage of incubation and declining as incubation time progressed. The *rub*A expression level of V_2 was down-regulated by the factor 10.115 as compared to that of LT_1. Despite the down-regulation, expression levels for *rub*A of V_2 were not significantly different from *rub*A of LT_1 with *p* values of 0.066.

During the amplification of *alk*M of V_2 samples, the *alk*M primer pair concurrently amplified the anticipated *alk*M gene (designated as *alk*M1 as shown in Figure 3A with a T_m of 80.36°C) as well an additional product; presumed to be another alkane hydroxlase that was tentatively designated *alk*M2 exhibiting a much higher T_m exceeding 92°C (Figure 3B) that was evident between 20 and 50 days of incubation. Amplification of *alk*M_1 and *alk*M_2 genes of V_2 samples was repeated and results were highly reproducible. Agarose gel electrophoresis of *alk*M_1 amplicons from LT1 confirmed the presence of the anticipated product of 189 bp from day 5 to 60 (Figure 3Ca). An additional product of 336 bp was evident (Figure 3Cb) from day 20 to 50 of V_2 samples which coincided with the melting curve analysis of *alk*M gene. The *alk*M gene amplified products of the V_2 at day 15 and LT_1 were sequenced and aligned. Partial sequence alignment (Figure 3D) shows 100% homogeneity of these two sequences. The *alk*M gene of LT_1 samples is therefore analogous to *alk*M_1 of V_2 isolate. Additionally, the obtained sequences showed 99 and 95% homogeneities with the partial sequences of alkane monoxygenase (*alkB*) from *Acinetobacter* sp. 49A and *A. calcoaceticus* PHEA-2, respectively. Sequencing of the day 20 sample of *alk*M_2 gene from isolate V_2 proved to be unsuccessful even after numerous attempts.

Formation of additional products could also be attributed to co-amplification of two or more genes as a result of sequence similarity. The presence of multiple alkane hydroxylases in a single bacterial strain is not an atypical occurrence (Tani et al., 2001; Marín et al., 2003).







(D)

V_2-15 5'-ATTCACGTCTTGTGAAGAGTTTTATTCCACTGCAATATGCTGCAAATGTTTATGCATGTT

LT_1 5'-ATTCACGTCTTGTGAAGAGTTTTATTCCACTGCAATATGCTGCAAATGTTTATGCATGTT

49A *alkB5* 5'-ATTCACGTCTTGTGAAGAGTTTTATTCCACTGCAATATGCTGCAAATGTTTATGCATGTT

PHEA-2 5'-ATTCACGTCTTGTGAA**A**AGTTTTATTCCA**T**TGCAATATGCTGCAAATGTTTATGCATGTT

V_2-15 5'-ATTTAACAAGCCGTAAAACCACGTCATTCATTGATAAAATTTTGCTCGGTGTTTCGATG(

LT_1 5'-ATTTAACAAGCCGTAAAACCACGTCATTCATTGATAAAATTTTGCTCGGTGTTTCGATG(

49A *alkB5* 5'-ATTTAACAAGCCGTAAAACCACGTCATTCATTGATAAAATTTTGCTCGGTGTTTC**A**ATGG

PHEA-2 5'-ATTTAACAAGCCGTAAAAC**T**ACGTCATTCATTGATAAAATTTTGCTCGGTGTTTCAATGG

Figure 3. Melting curve analysis of *alk*M gene of (A) LT_1 and (B) V_2 samples, (Ca) Electrophoresed (2%) *alk*M1 gene amplicons of LT_1A. Lanes 1, Molecular weight marker IV (Roche); 2, negative control (no cDNA); 3, positive control; 4 - 10, day 5 to 60, respectively. (Cb) Electrophoresed *alk*M1 and *alk*M2 gene amplicons of V_2 samples --Lanes 1, Molecular weight marker IV (Roche); 2, negative control (no cDNA); 3, positive control; 4 - 11, days 5 to 60, respectively. (D) Partial sequence alignment of *alk*M1 amplicons of LT_1, V_2 and partial sequences of alkane monooxygenase of *A. calcoaceticus* PHEA-2; *Acinetobacter* sp. 49A.

Table 3. Descriptive statistics and variation data output for target genes of LT_1, and V_2 samples.

Factor	16S rRNA	*rub*A	*alk*M	*alk*R	*lip*A	*lip*B	*xcp*R	*est*B
LT_1								
n	7	7	7	7	7	7	7	7
Sample means	12.27	25.46	32.14	39.48	-	40.94	34.58	-
Min	10.32	23.02	29.7	35.46	-	37.08	31.98	-
Max	13.56	28.55	35.57	44.50	-	46.30	37.90	-
SD	1.27	3.55	4.06	10.11	-	10.27	3.63	-
CV (%)	10.31	13.92	12.65	25.60	-	25.10	10.49	-
V_2								
n	8	8	8	8	8	8	8	8
Sample means	12.29	29.09	*	*	-	39.76	*	31.81
Min	9.542	23.84	*	*	-	37.70	*	29.34
Max	16.86	34.50	*	*	-	41.23	*	33.58
SD	6.61	10.48	*	*	-	1.41	*	2.38
CV (%)	53.81	36.02	*	*	-	3.55	*	7.47

- Invalid C_p values due to nonspecific binding; * invalid C_p values due to multiple product formation. Analysis of 16S rRNA gene expression.

Multiple alkane hydroxylases enable alkane degraders to grow on a broad range of alkane substrates, where each alkane hydroxylase may exhibit unique properties and have different induction patterns (van Beilen et al., 2003). The *alk*Ma and *alk*Mb expressions of *Acinetobacter* sp. M1 were differentially induced in response to *n*-alkane chain length (van Beilenet al., 2003). Differential expressions of *alk*B1 and *alk*B2 of *P. aeruginosa* were not based on alkane chain length but rather on the levels of oxygen (Marín et al., 2003). In addition to two genes encoding AlkB-type alkane hydroxylase homologues in *Acinetobacter* sp. strain DSM 17874, *almA*, which encodes a putative flavin-binding monooxygenase, was also involved in the metabolism of long-chain n-alkanes (Throne-Holst et al., 2007). The *A. venetianus* VE-C3 genome possesses different alkane degrading genes which encode AlkB-like, soluble cytochrome P450 monooxygenases, AlmA and LadA (Fondi et al., 2013). V_2 might have been evolved to possess at least two alkane hydroxlases/monooxygenases to cope with the stress of the longer carbon chain length and/or under oxygen levels as *alk*M1 was expressed at the late exponential/early stationary phase and *alk*M2 throughout the late stationary phase.

In compliance with multiple *alk*M gene expressions, multiple *alk*R and *xcpR* genes of V_2 samples were also expressed to enhance the regulation of *alk*M expression (Figure 4). The expected amplification product of *alk*R gene in LT_1 samples, was only observed at late stage of degradation (days 30, 50, and 60) and in the V_2 samples, and there was shift in T_m of approximately 2°C for day-30 and 60 samples (Figure 4A). The presence of a second product (*alk*R2) with T_m exceeding 84°C was also observed at the early stage of diesel degradation (days 5, 10 and 15 samples). Despite the disparity in T_m, the amplification product size was identical to that of day 50. No *alk*R amplification occurred for days 20 and 40 samples. The *alk*R_1 gene of V_2 might be analogous to *alk*R of LT_1 on the basis of T_m and amplification product size. *A. calcoaceticus* sp. M1 that contained the two alkane hydroxylases *alk*Ma and *alk*Mb was also found to possess two transcription regulators *alk*Ra and *alk*Rb (Tani et al., 2001). Each alkane hydroxylase was regulated by either *alk*Ra or *alk*Rb responded to the alkane chain lengths. The *alk*R_1 gene of V_2 is comparable to *alk*R of LT_1 on the basis of T_m and amplification product size.

The *xcpR* gene, that encodes a subunit of the general secretory pathway, is required for alkane degradation in *Acinetobacter* sp. ADP1 and DSM 17874 strains (Parche et al., 1997; Throne-Holst et al., 2007). The expression of *xcp*R in conjunction with *rub*A and *rub*B in *A. calcoaceticus* is known to be constitutive (Ratajczak et al., 1998). Figure 4B shows that the presence of additional amplification products for *xcp*R of V_2 samples, illustrated by three defined peaks, exhibited higher T_ms. The results obtained for V_2 samples were highly reproducible yielding multiple products with identical melting temperatures. Real-time PCR amplification products of *xcp*R of V_2 samples were verified by gel electrophoresis (Figure 5). The presence of two distinct products was observed. The expected 192 bp product (*xcp*R1) was observed in all samples and the presence of a larger fragment of approximately 500 bp (*xcp*R2) was also observed in all samples except on days 30 and 40. The metabolism of *n*-alkanes in the V_2 isolate appears to be more complex. Its

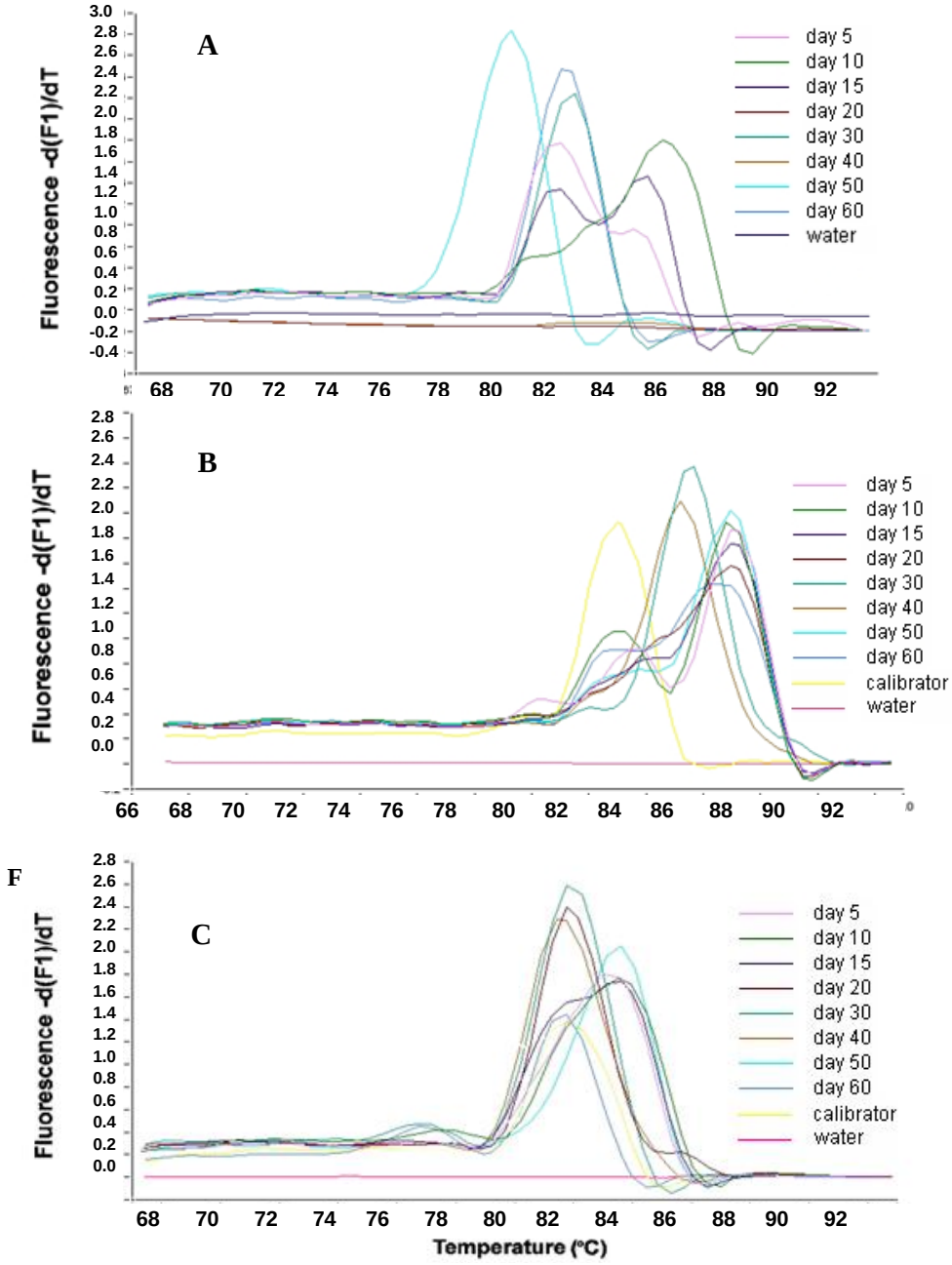


Figure 4. Melting peak analysis of (A)*alk*R gene,(B) *xcp*R and (C) *lip*B gene of V_2 samples determined by plotting the negative derivative of fluorescence [-d(F1)/dT].

broad substrate range, diversity and functions of the enzymes involved in alkane degradation, and its contrasting regulation of both constitutive and differential gene expression may be reminiscent of the fact that two and perhaps more *xcp*R genes are required to provide an adequate transport system during alkane degradation.

Rubredoxin (*rub*A) activity was detected in all investigated samples of LT_1 and V_2 isolates. LT_1 and V_2 samples exhibited similar *rub*A expression trends and the differences in expression levels were not significant. However, rubredoxinreductase (*rubB*) activity was not detected in any of the isolates despite rigorous optimising strategies and redesigning of primers. Plausible reasons for the inability to detect *rub*B activity could be attributed to very low expression levels, or poor primer design.

Theoretically, *rub*B would have exhibited a similar

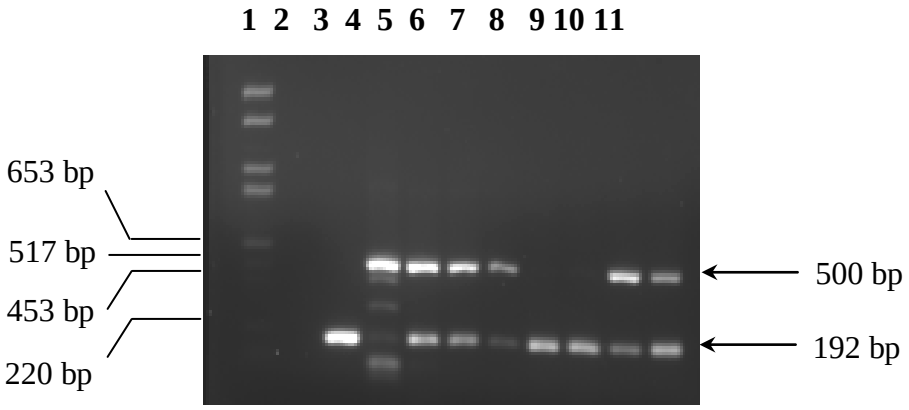


Figure 5. Electrophoresed *xcp*R amplicons of V_2 samples. Lanes 1- molecular weight marker VI (Roche); 2- negative control (no cDNA template); 3- positive control (calibrator); 4 to 11- *xcp*R amplicons of 5 to 60 days samples, respectively.

expression trend to that of *rub*A due to their synergistic association (Geißdörfer et al., 1999). Interestingly, novel genes encoding AlkB-Rubredoxin fusion proteins were used in the hydroxylation of long-chain alkanes by Gram positive bacteria (Nie et al., 2011). In addition, rubredoxin-rubredoxin reductase systems are present in many other organisms that are unable to degrade alkanes, where they serve other functions such as rapid transport of reducing equivalents to the final receptor (Hagelueken et al., 2007).

Although, *rub*A, *rub*B and *est*B constitute an operon in *Acinetobacter* sp. ADP1, it was established that no association could be ascertained between the expression of *rub*A and *est*B in accordance with the findings by Geißdörfer et al. (1999). The expression of *est*B was predominantly observed in V_2 samples and either non-expression or extremely low expression of the *est*B gene in LT_1 samples (Figure 3). During the diesel degradation phase of V_2 inocula, an opaque, waxy material was observed, probably due to bioemulsifiers in the hydrocarbon and hydrocarbon-culture medium fraction, but not in flasks containing LT_1 inocula. These findings are suggestive that the involvement of *est*B in the release of the bioemulsifier may be plausible (Shabtai and Gutnick, 1985).

Low expression of *lip*A in LT_1 and lack of expression in V_2 (Table 3) is an indication of its non-involvement in the degradation of diesel. The *lipB* gene was expressed in all samples in this study. Expression of *lip*B amongst V_2 samples was moderately constant exhibiting lower SD and CV% values of 1.41 and 3.55, respectively (Table 3). The expression profile of *lip*B gene in V_2 (Figure 4C) was found in low level, depicted by the high C_p values exhibiting maximum expression at day 15. Thereafter, expression levels declined fairly constantly. In comparison to LT_1, *lip*B of V_2 was up-regulated by the factor 1.976 with no significant difference (p= 0.436). In this study, the expression trends of *lipA* and *lipB* were incongruent, and expression of *lipB* was still evident in the absence of *lipA* expression amongst V2 samples. The *lipB* gene encodes a lipase chaperone that prevents the complete folding of the lipase before it traverses the outer cell membrane (Kim et al., 2008). Alternatively, steric chaperones may have other functions such as the autotransporter protein that can be used to direct a variety of proteins to the cell surface in addition to LipA folding (Wilhelm et al., 2007). Kim et al. (2008) also reported that there are 3 different *lipa*A genes present in *Acinetobacter* sp. DYL 129. The involvement of *lip*A *and lip*B in alkane degradation is still unknown.

The findings of this study confirm the importance of *rubA, alkM, alkR,* and *xcpR* genes in the process of alkane degradation. The results are also suggestive that different members of the alkane degraders, such as *Acinetobacter* LT_1 and V_2 strains in this study employ a variety of alkane degrading pathways for alkane oxidation, albeit both isolates achieved comparable levels of diesel degradation. It is apparent that alkane oxidation in V_2 appears to be complex due to its diverse regulations of multiple alkane hydroxylases, alkane hydroxylase transcription regulators and the secretory pathways. However, involvement of other alkane degrading proteins such as cytochrome P450 alkane hydroxylases, Lad and AlmA cannot be ruled out.

Conclusions

We concluded that both *Acinetobacter* strains LT_1 and V_2 in this study showed comparable diesel degrading ability, however real-time PCR experiments revealed more than one enzyme system involved by V_2 indicating its diverse regulations of multiple alkane hydroxylases, its transcription regulators and the secretory pathways. Low or no expression of *lipB* and *lip*A genes suggested the absence

of lipases in degradation. This study also confirms the importance of *rubA, alkM, alkR* and *xcpR* genes in the process of alkane degradation, although, future experiments would be necessary to elucidate the involvement of other proteins such as cytochrome P450 alkane hydroxylases, Lad and AlmA in alkane degradation.

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The dynamics of bacterial population during growth and decomposition of phytoplankton in a tropical productive pond water ecosystem

Raphael Teken FORTU* and Ekpo Eyo ANTAI

Department of Freshwater Conservation, Brandenburg University of Technology, Cottbus, Germany.

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In this study, a field experiment was set up to examine the seasonal dynamics of bacterial population and to investigate the relationship between bacterial abundance and dissolved organic carbon (DOC) produced by phytoplankton in a tropical eutrophic fishpond located in Calabar, South-East Region of Nigeria. Sampling lasted for six months: January to March in the dry season and April to June in the wet season. The highest concentration of DOC released during the dry season was 4.440 mg/L in January, while during the wet season; the highest concentration of 4.992 mg/L was released in April. The patterns of nutrients (nitrate and phosphate) released during both seasons were similar. In the dry season, the range of bacterial count varied between 7.307 x 10^4 and 0.025 x 10^4 CFU/ml while during the wet season it was from 7.909 x 10^4 to 0.019 x 10^4 CFU/ml. The bacteria; *Vibrio, Moraxella* and *Bacillus* species were dominantly present throughout the dry season but during the wet season, the bacteria; *Pseudomonas*, *Flavobacterium, Streptococcus, Bacillus, Corynebacterium* and *Aeromonas* species dominated throughout. Bacterial species succession also took place within seasons. Our results suggest that, during growth and decomposition of phytoplankton, the bacterial community involved shows a successive change not only in generic composition but also in terms of the heterotrophic activity of the bacteria in the community. The correlation analysis between dissolved organic carbon and bacterial count showed a positive correlation (r = 0.95 for the dry season and r = 0.82 for the wet season). There was a significant difference ($p<0.05$) between season in the case of DOC and bacterial count.

Key words: Dissolved organic carbon (DOC), bacteria, nutrient, species, and succession.

INTRODUCTION

Dissolved organic carbon (DOC) is one of the largest reservoirs of reactive carbon in pond water, which play crucial roles in the global geochemical cycle of carbon. The major source for DOC in pond water is biological and related to primary productivity (Tang et al., 2009). Nutrient inputs to lakes, ponds, etc are often dominated by nutrient release from sediments. Rates of nutrient release from the decomposition of sedimentary organic matter such as dead phytoplankton are determined by bacterial demands for food and energy. During phytoplankton growth, dead and decomposition in the pond, primary productivity and its processing by the food web create a heterogeneous environment of particulate, colloidal, and dissolved organic matter in a continuum of size classes and concentrations (Azam, 1998). Major changes in organic matter concentration and composition are expected to occur at different stages of phytoplankton growth and decomposition. The variations in the organic matter regime are typically accompanied by pronounced changes in bacterial abundance, productivity, ectohydrolase activities, and colonization of particles (Smith et al., 1995). For instance, dominance of bacteria

*Corresponding author. E-mail: raphaeltekenfortu@yahoo.com.

specialized in colonizing and hydrolyzing phytoplankton detritus would greatly influence the biogeochemical transformation of the detritus. At the same time, free-living species adapted for efficiently utilizing dissolved organic matter could prevent large-scale dissolved organic matter accumulation despite its high production rates typical in phytoplankton growth and bloom (Koike et al., 1990; Smith et al., 1995). Van Hannen et al., (1999) showed that the origin of detritus can indeed affect the structure of the bacterial community.

However, a number of studies have also demonstrated that flagellate grazing has the potential to affect the species composition of a mixed bacterial community. The extent of flagellate grazing of bacteria is affected by bacterial size and motility (Gonzalez et al., 1993; Pinhassi et al., 2004; Pisman et al., 2005; Simek et al., 1992), and protozoa consume and digest various bacterial species with variable efficiencies (Gonzalez et al., 1990). Furthermore, Caron (1987) showed a strong specialization among flagellate species in the ability to graze free-living and attached bacteria and Simek et al. (1997) observed a shift in the numerical dominance of different subdivisions of the class *Proteobacteria* as a consequence of heavy flagellate grazing. However, studies that simultaneously measure changes in the bulk biochemistry and phylogenetic composition of the bacterial community are just beginning to evolve (Rooney-Varga *et al.,* 2005).

The present study was designed to investigate the seasonal fluctuation of the number and type of bacteria, dissolved organic carbon and inorganic nutrients (nitrate and phosphate) during growth and decomposition of phytoplankton in a productive pond water ecosystem. It also attempted to investigate the relationship between bacterial abundance and DOC that may enhance these observed dynamics in bacterial population.

MATERIALS AND METHODS

Study area

The University of Calabar (Unical) fish farm is located in the vicinity of the University of Calabar staff quarters at approximately 04.56°, 020'N and 08.20°, 456'E in Cross River State, Nigeria. The climate of the area is governed by its latitude and to a large extent by the two dominant winds, the Southeast monsoon and the northeast trade winds common in most of West Africa. The area is also characterised by distinct wet and dry seasons. Mean annual rainfall is about 2000 mm and temperatures generally range from 22°C in the wet to 35°C in the dry season (Akpan and Offem, 1993).

Experimental design

The field experiment was mounted at the University of Calabar fish farm. Three tanks, each of about 3000 L volume capacity (two experimental tanks and one control tank) were filled with pond and bore-hole water respectively. The experimental tanks were each pre-treated with 100 g of the fish pond fertilizer 20-20-0 as additional source of nutrient to facilitate phytoplankton growth and bloom. This was necessitated by the low levels of nutrients initially measured (0.01 mg/L for phosphate and 0.4 mg/L for nitrate). The tanks were left to stand for two days exposed to natural environmental conditions before sampling. Just prior to sampling, the tanks were gently stirred with a polyvinyl chloride pipe to resuspend any particulates accumulating around the bottom edges of the tanks. Sampling was accomplished by siphoning 1 L of water from the center of each tank with silicone tubing into polycarbonate carboys. All variables were daily monitored. Aliquots for bacterial count were fixed immediately after sampling, and identification was initiated thereafter. Samples for nutrients and DOC were processed and then immediately frozen for later analysis.

Phytoplankton enumeration and identification

Bulk samples of 50 mL were fixed with Lugol's solution in sedimentation chambers (2.5-10 mL) and cells were enumerated with an inverted microscope according to Utermohl technique as reported by Edler and Elbrachter (2010). Phytoplankton species were identified with the help of taxonomic catalogue proposed by Botes (2003).

Determination of dissolved organic carbon (DOC)

The high temperature combustion method outlined by Bruckner (2011) was used for DOC measurement. According to this method, sample preparation and processing protocol is outlined as follows:

1) The sample was collected in a glass container that was baked in the laboratory at 550°C for 2-4 h (the baking process removes any residual carbon in or on the collection vessel that may cause contamination); 2) A known volume of sample (1000 mL) was filtered through a 0.22 µm pore size nitrocellulose filter with the aid of the vacuum pump. Filters were cleaned by passing deionised water through them before collection to prevent DOC leaching: 3) The filtered samples were combusted at high temperature which involves conversion of inorganic carbon to dissolved CO_2, and purging this from the sample and 4) the remaining (organic) carbon was then oxidized at a high temperature to CO_2 which was detected by the instrument's non dispersive infrared (NDIR) sensor and directly correlated to total organic carbon (TOC) content.

Nutrient analysis

Water samples were filtered through 0.45 µm pore size membrane filters and preserved by freezing at – 10° C.

Determination of phosphate concentration

The Molybdenum blue method reported by Kolo et al. (2010) was used. 5 ml of mixed reagents (mixture of ammonium molybdate solution, sulphuric acid solution, ascorbic acid solution and potassium antimonyl tartrate) in the ratio 1:2.5:1:0.5 was added to 50 ml of the sample and allowed to stand for 30 minto 1 h for colour development. It was read at 885 nm with spectrophotometer.

Determination of nitrate concentration

The Cadmium reduction method of Parsons et al. (1984) was used. The cadmium was shaken several times in copper sulphate solution. Slurry was used to pack the column. The column was washed several times with ammonium chloride buffer. The column was drained and 100 ml of sample was allowed to pass through it. The initial 25-30 ml was discarded. 50 ml was collected from the

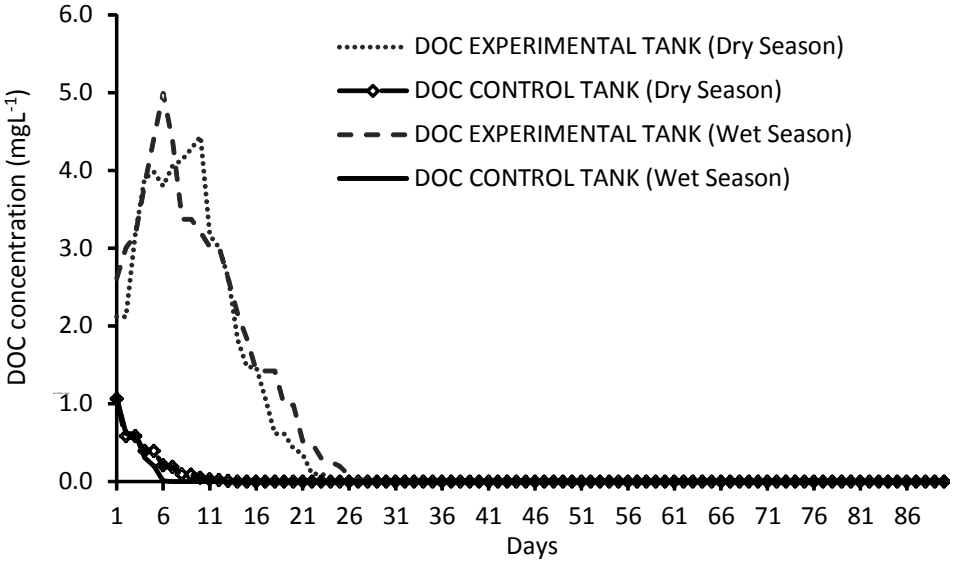

Figure 1. DOC concentrations (mgL^{-1}) during dry and wet seasons.

column as reduced sample used for the analysis. 2 ml of sulphonilic acid was added to the 50 ml reduced sample. It was thoroughly mixed and after 2 to 8 min, 1 ml of naphthylethylene diamine (NED) was added. After 20 min, the concentration was measured at 540 nm against blank.

Enumeration and identification of bacteria

Bacterial cell numbers were counted by staining with 4', 6 – Diamidino - 2 - phenylindole followed by Epifluorescence Microscopy according to the procedure of Porter and Feig (1980). Bacterial cells were also grown on nutrient agar medium by the pour plate method. Isolated colonies were subjected to morphological characterization, differential staining and biochemical testing and, identified to genus level with the aid of Bergey's manual.

Statistical method

Correlation and regression analysis was carried out between dissolved organic carbon and bacterial count.

RESULTS

Phytoplankton abundance

Phytoplankton species richness and successions were observed during both seasons with the class Chlorophyceae (>99%) predominantly present throughout both seasons (data not shown). The phytoplankton community was dominated by *Crucigenia lauterbornei, Selenastrum gracile, Chlorella vulgaris, Gloeobotryls limnetica,* and *Dinema griseolum* during the dry season and by *Scenedesmus dentriculatus, Lobocystis dichotoma, Scenedesmus quadricauta, Goeobotryls limnetica* and *Euglenopsis vorax* during the wet season (data not shown).

DOC concentrations

The DOC concentrations increased up to day 10 and day 6 of the dry and wet seasons respectively to maximum and then started to decline in the experimental tanks. The DOC concentrations in control tanks decreased continuously from onset. As can be seen in Figure 1, the highest concentration of DOC released by phytoplankton during the dry season was 4.440 mg/L, while a higher concentration of 4.992 mg/L was released during the wet season.

Nutrients patterns

The patterns of both nutrients (nitrate and phosphate) analysed during the dry and wet periods were similar. In all cases, a slight increase in nutrient concentration was observed on day 2 of sampling. It then decreased, increased later and then decreased to the end. Also, higher values in nutrient concentrations were observed during the wet season than the dry season (Figures 2a and b).

Bacteria abundance

The fluctuation patterns for bacterial abundances were very similar during both seasons. The highest count of 7.307×10^4 CFU/ml and lowest count of 0.025×10^4 CFU/ml were observed on days 4 and 17 respectively during the dry season. Much higher count of 7.909×10^4 CFU/ml and lowest count of 0.019×10^4 CFU/ml were observed on days 3 and 85 respectively during the wet season. Generally, much higher values of bacterial count were observed during the wet than the dry season (Figure 3).

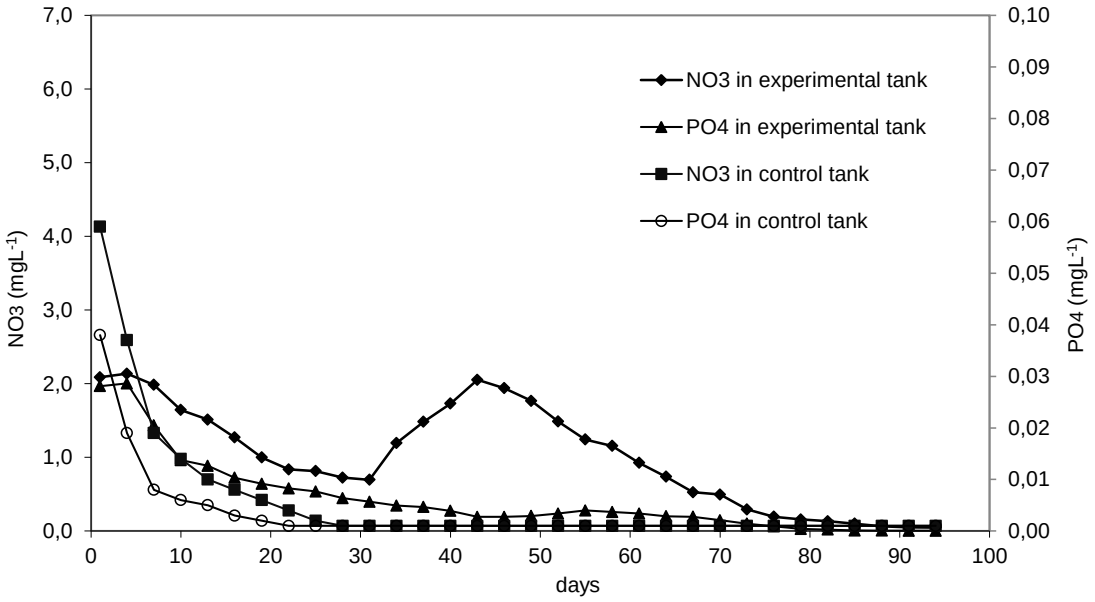

Figure 2a. Nutrient concentrations during the dry season (January to March).

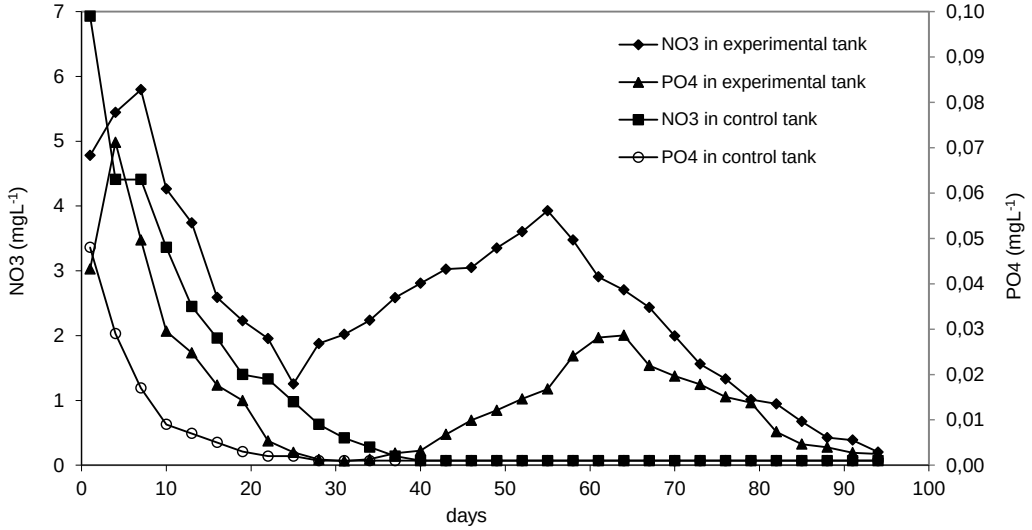

Figure 2b. Nutrient concentrations during the wet season (April to June).

Bacteria species

From biochemical characterization and identification, the bacteria; *Vibrio spp., Moraxella spp* .and *Bacillus spp.* were predominantly present throughout the dry season. The early part of this season was dominated by *Pseudomonas spp.*, *Flavobacterium spp.* and *Micrococcus spp.*, while the later part was dominated by *Corynebacterium spp* and *Streptococcus spp.* (Figure 4a). But during the wet season, the bacteria; *Pseudomonas spp., Flavobacterium spp., Streptococcus sp.p, Bacillus spp., Corynebacterium spp.* and *Aeromonas spp.* were predominantly present throughout. The early part of this period was dominated by *Vibrio spp., Micrococcus spp.* and *Moraxella spp.* (Figure 4b).

Correlation and regression analysis

The correlation analysis between dissolved organic carbon and bacterial count shows a positive correlation between these two variables with linear correlation coefficients (r) of 0.95 and 0.82 for the dry and wet seasons respectively and that, there is a high significant difference (p = 0.05) between DOC and bacterial count during both seasons.

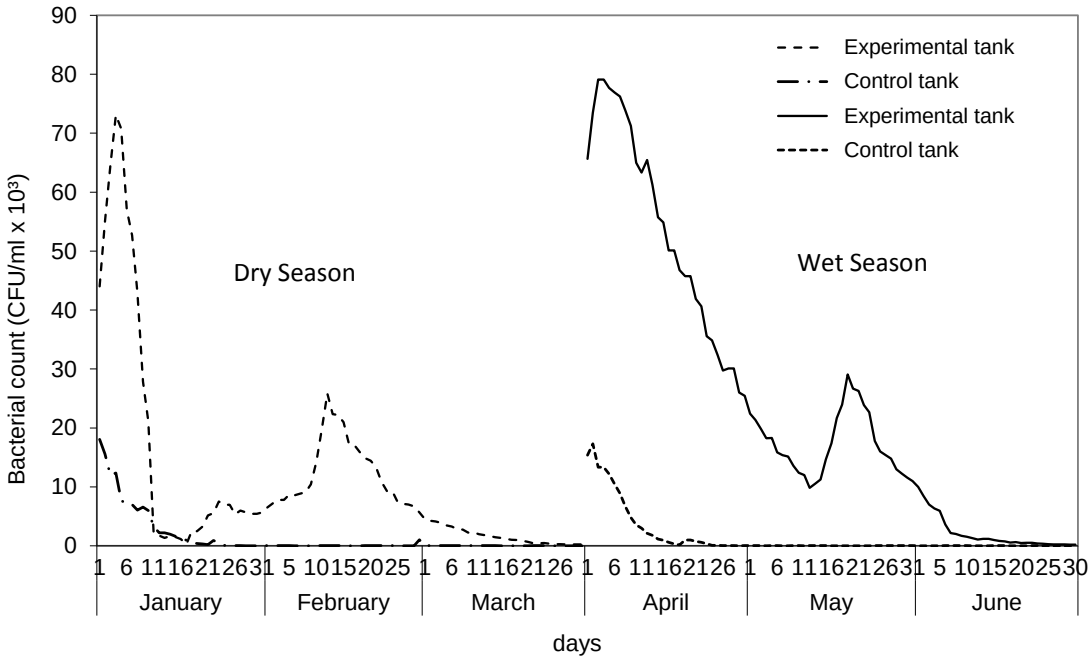

Figure 3. Heterotrophic bacteria distribution during dry and wet seasons.

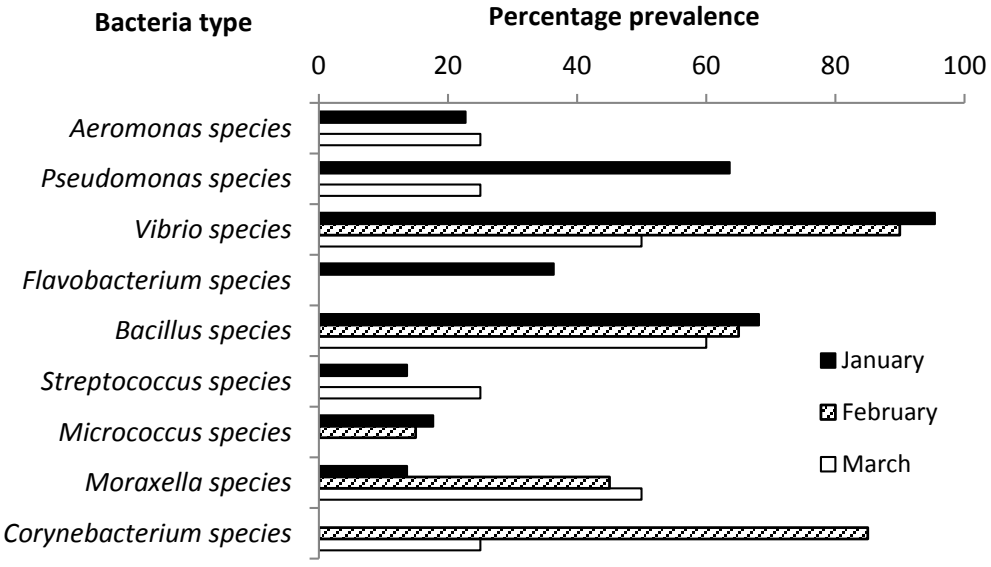

Figure 4a. Percentage prevalence of bacterial species isolated during the dry season.

DISCUSSION

DOC was released mainly through excretion and lysis of phytoplankton and it had a dramatic increase from on set and then decreased later throughout both seasons. The highest concentrations observed (4.440 mg/L for dry season and 4.992 mg/L for wet season) correspond to phytoplankton growth peak where maximum bloom occurs (Figure 1). Bloom may continue until nutrients are exhausted or grazing balances production (Maddocks, 1998). Brussaard (2004) reported that through cellular lysis, viruses indirectly affect the fluxes of energy, nutrients, and organic matter, especially during phytoplankton bloom events when biomass is high. The decreasing phase in DOC corresponds to the dead and decomposition of phytoplankton due to nutrient exhaustion and grazing by zooplankton, and also due to certain chemical substances produced by certain bacteria which show antimicroalgal

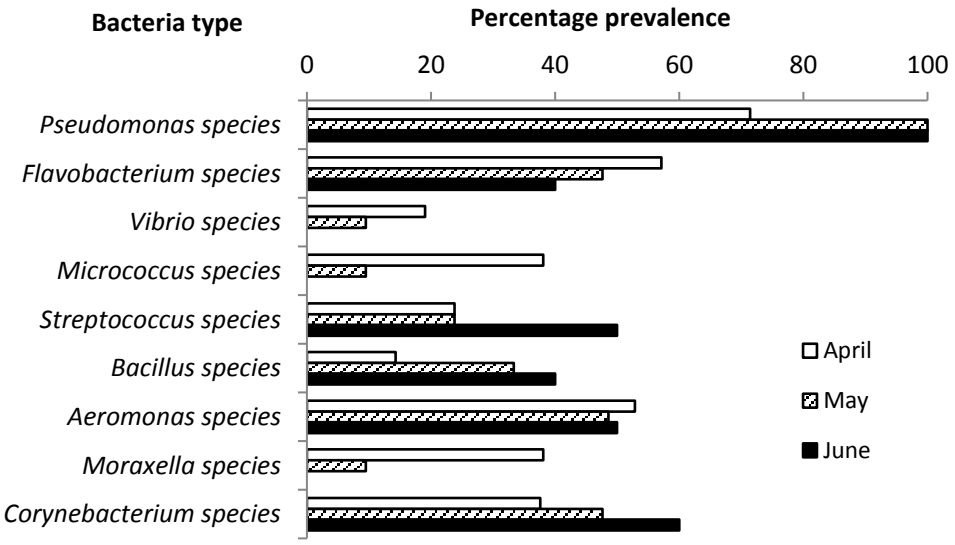

Figure 4b. Percentage prevalence of bacterial species isolated during the wet season.

activity (kawano et al., 1997; Ichiro-lmai, 1997). Tang et al. (2009) reported that this decrease resulted from the absorption of DOC by bacteria.

The periods of increase in nutrients (Figures 2a and b) could be explained by the continuous action of bacteria on dead phytoplankton cells, thereby unlocking or regenerating nutrients for re-use by a new generation leading to succession. The decrease observed in nutrient concentration during both seasons was due to increasing competition by the relatively increasing phytoplankton and bacterial population (Zevenboom and Westeyn, 1990), and also since the phytoplankton dead cells produced at the surface do not decompose in the euphotic zone but reaches greater depths before decomposition, thus transporting nutrients from the surface to deeper water masses. The rate at which this enriched water returns to the surface for re-utilization is very slow (Maddocks, 1998). Nutrient inputs to ponds are often dominated by nutrient release from sediments. Schultz and Urban (2007) explained that rates of nutrient release from the decomposition of sedimentary organic matter are determined by bacterial demands for food and energy.

During growth and decomposition of phytoplankton, fluctuations in the number of heterotrophic bacteria showed trends similar to those reported previously (Fukami et al., 1985). The heterotrophic bacterial count increases sharply from onset to maximum values of 7.307×10^4 and 7.909×10^4 CFU/ml during the dry and wet seasons respectively (Figure 3).This first phase of increase to maximum value was due to the presence of DOC released by the growing phytoplankton, in addition to nitrate and phosphate in the water, which were all utilized by the bacterial community for growth. The second period of slight increase in bacteria count observed was due to nutrient regenerated from dead phytoplankton cells by the decomposition activity of bacteria and according to Riemann et al. (2000), during this phase, attached bacteria could be one or two order of magnitude more than free - living bacteria. There are specific interactions between phytoplankton and the bacteria attached to them, and these interactions influence the composition of both communities (Rooney-Varga *et al*., 2005). Such interactions increase aggregate formation and particle sinking and thus may enhance the efficiency of the biological pump (Gardes et al., 2011). The decreasing phases observed during both seasons were due to the decreasing nutrient concentration and grazing by zooplankton. Tijdens et al. (2008) explained that such temporal changes in bacterial abundances could be significantly related to viral community assemblage, and vice versa, suggesting an interaction between viral and bacterial communities.

Bacterial species succession was observed from month to month within both seasons and from season to season as shown in Figures 4a and b. This is in conformity with some recent studies which have demonstrated bacterial succession both seasonally in the field (Riemann et al., 2008; Tian et al., 2009). Riemann et al. (2000) reported that, the observed changes could have resulted from community succession with bacteria with inherently different metabolic capabilities predominating at different stages of phytoplankton growth and decomposition. Species successions could then affect as well as reflect bacterial-organic matter coupling and biogeochemical fate of phytoplankton growth and decomposition.

From correlation and regression analysis, there exist a positive correlation ($r = 0.95$ for the dry season and $r = 0.82$ for the wet season) and a high significant difference ($p = 0.05$) between DOC and bacterial count during both seasons indicating that, as DOC increases so do bacterial count. This suggests that the DOC produced by phytoplankton through excretion and lysis was the main

source of carbon for the bacterial community. Although potentially toxic phytoplankton would have been involved, they have been shown to produce DOC which was accessible to the bacterial community for growth (Armando, 2001).

Conclusions

This study shows that, growth and decomposition of phytoplankton in a tropical productive pond water ecosystem is accompanied by major changes in bacterial community composition. It further shows the highly dynamic nature of bacterial community composition and strongly suggests that nutrient-induced changes in natural phytoplankton communities lead to significant effects on the structure and functioning of bacterial assemblages as well as on the nature and the rates of bacterially mediated organic matter cycling. This study further emphasises the need to incorporate community composition into our conceptual thinking of the biogeochemical activities of aquatic microbial assemblages.

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African Journal of Microbiology Research

Full Length Research Paper

Efficacy of aminocyclopropane-1-carboxylic acid (ACC)-deaminase-producing rhizobacteria in ameliorating water stress in chickpea under axenic conditions

Palika Sharma, Veena Khanna* and Poonam Kumari

Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana 141004, India.

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To mitigate environmental stresses, use of aminocyclopropane-1-carboxylic acid (ACC)-deaminase containing plant growth promoting rhizobacteria (PGPR) as agricultural inputs for improved crop production is required. A total of 47 bacterial isolates from different rhizospheric soils of chickpea from Punjab were biochemically characterized and found to be representatives of genus *Bacillus* (25) and *Pseudomonas* (22). Ten (10) of the isolates were able to utilize ACC as a sole source of nitrogen, maximum growth (in terms of optical density λ600) being recorded with *Bacillus* isolate 23-B (0.463) followed by *Pseudomonas* 6-P (0.317). Three isolates were P-solubilizers and their relative P-solubilization efficiency ranged from 14.6 to 21.6 mg/100 ml culture broth. All the isolates produced Indole-3-acetic acid (IAA) (4.4-22.8 µg/ml). Two PGPR's 23-B and 6-P alone and in combination with recommended (for Punjab state) *Mesorhizobium ciceris,* were evaluated for water stress mitigation and plant growth promotion under axenic conditions on *Cicer arietinum* varieties (*Kabuli* L-552 and *Desi* GPF-2). Both the rhizobacteria significantly improved germination, root and shoot length and fresh weight of chickpea seedlings under osmotic potential of up to 0.4 MPa over uninoculated control. Proline content was considerably higher in PGPR treated varieties of chickpea under water stress. Co-inoculation of 23-B with *Mesorhizobium* enhanced all growth parameters under water stress.

Key words: Aminocyclopropane-1-carboxylic acid (ACC)-deaminase, axenic, chickpea, polyethylene glycol (PEG), proline.

INTRODUCTION

Abiotic stresses caused by complex environmental conditions, for example, drought, salinity, high and low temperatures etc lead to substantial crop losses worldwide (Mittler et al., 2006). As a consequence of these stresses, plants typically stimulate 1-aminocyclo-propane 1-carboxylic acid (ACC) synthesis, a precursor to ethylene (Gamalero and Glick, 2012) which helps in inducing multifarious physiological changes in plants at molecular level but at higher levels is usually deleterious, as it induces defoliation, changes cellular processes leading to growth inhibition, premature senescence, restricted nodulation, all of which reduce crop yield (Lie et al., 2005). Amongst the legumes, chickpea (*Cicer arietinum* L.) is third most important grain legumes in the world cultivated on 11.55 million hectares with production of 10.46 million tons, India being the largest producer

*Corresponding author. E-mail: veenadk@rediffmail.com.

(FAO STAT, 2010). To reduce the deleterious effects of ethylene stress, plant growth-promoting rhizobacteria (PGPR) that facilitate the proliferation of plants under stress conditions are a potentially viable option (Gamalero and Glick, 2012). These beneficial rhizobacteria with ACC deaminase activity sustain plant development by lowering ethylene levels by metabolizing ACC into α-ketobutyrate and ammonia (Mehta et al., 2010).

Under stress conditions, increased proline biosynthesis has been reported in various plant species inoculated with different PGPR (Vardharajula et al., 2011). Proline acts as osmoprotectant and reactive oxygen species scavenger thus supporting plant growth under stress. ACC-deaminase activity has been widely reported in numerous species of PGPR like *Azospirillum*, *Bacillus, Burkholderia, Pseudomonas* and *Rhizobium* etc (Shaharoona et al., 2006).

The result of adding PGPR to plants is a significant increase in seed germination and the biomass that the plants are able to attain under otherwise stressful and inhibitory conditions (Shaharoona et al., 2012). The present work was undertaken with the objective to screen rhizobacteria producing ACC-deami-nase for plant growth promoting potential under water stressed conditions.

MATERIALS AND METHODS

Isolation and characterization of bacterial strains

Soil samples were collected from the depth of 10 to 15 cm from twenty different chickpea fields of Punjab. The soil samples were serially diluted up to 10^{-9} dilutions and were plated on nutrient agar (for *Bacillus* spp.) and King's B (*Pseudomonas* from spp). The plates were then incubated at 30°C for 24 h.

The colonies were picked up, sub-cultured and preserved on Nutrient agar slants at 4 to 5°C. The isolates were then characterised on the basis of colony morphology, Gram staining and biochemical tests namely: catalase production, nitrate reduction, starch hydrolysis and methyl red test (Cappuccino and Sherman, 1992).

Aminocyclopropane-1-carboxylic acid (ACC)-deaminase activity

Bacterial isolates were grown in Luria Broth medium and cell pellet collected by centrifugation washed and resuspended in sterile water and spot inoculated on petriplates containing Dworkin and Foster (DF) salt minimal medium (Dworkin and Foster, 1958) supplemented with 3 mM ACC as a main source of Nitrogen.

In quantitative assay each selected isolate was grown individually in liquid DF minimal medium alone, DF+ACC and DF+ $(NH_4)_2SO_4$ and their growth measured (OD_{60}). ACC-deaminase producing rhizobacterial isolates were selected for evaluation of other PGP traits.

Phosphate-solubilization

Phosphate-solubilization was evaluated qualitatively (Mehta and Nautiyal, 2001) and solubilization index calculated

$$\text{Phosphate solubilization Index} = \frac{\text{Total diameter (colony + halo zone)}}{\text{Diameter of colony}}$$

Quantitatively P-solubilization was recorded at intervals of 3 days up to 15 days (Jackson, 1973).

Production of Indole-3-acetic acid (IAA)

Bacteria were grown in Luria broth (72 h at 30°C) and IAA estimated by the method of Gordon and Weber (1951).

Induction of water stress by polyethylene glycol 6000 (PEG)

Seeds of two *Cicer arietinum* varieties (L-552 and GPF-2) obtained from Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana, were surface-sterilized and soaked in rhizobacterial culture (23-B and 6-P showing relatively higher ACC-deaminase activity, P-solubilization, IAA synthesis and compatibility with *Mesorhizobium ciceris)* alone and in combination with *Mesorhizobium* culture for 30 min. The experiment was conducted in completely randomized design with three replications. To simulate drought stress, PEG-6000 equivalent to water potential -0.2, -0.4 and -0.6 MPa (Mega Pascals) were used. Ten seeds of each genotype were germinated on sterilized filter paper lined petri dishes, moistened with 5 ml solution of PEG-6000 having appropriate osmotic pressure and kept in incubator at 22 ± 2°C with 90% humidity for duration of 10 days. Seeds germinated using distilled water only served as absolute control.

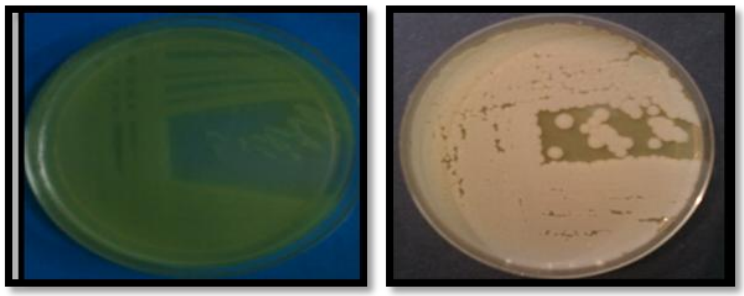
Proline content

Samples were extracted in Methanol: Chloroform: Water (12:5:1), centrifuged and the supernatant collected and made up to 10 ml with same solvent. To this 6 ml of chloroform and 4 ml of distilled water was added and allowed to stand till the two layers got separated. The final volume of upper layer was made 10 ml with distilled water, to 5 ml of this solution 2.5 ml of acid ninhydrin was added and mixture boiled for 45 min till pink colour developed and OD at 515 nm was recorded (Bates et al., 1973). Proline was used as standard to make standard curve (Sahu and Sindhu, 2011).

RESULTS AND DISCUSSION

Isolation and characterization of rhizobacteria

A total of 47 rhizobacteria were isolated from chickpea rhizospheric soils and out of these 22 were from Kings B medium, all showed yellowish green pigment characteristic of *Pseudomonas* spp whereas 25 were from nutrient agar medium showed predominantly off-white to creamish colonies, typical of the genus *Bacillus* (Figure 1). The predominance of *Pseudomonas* and *Bacillus* spp. in legume rhizosphere has been reported by many workers (Yadav et al., 2010). Most of the *Bacillus* isolates were indole and citrate negative, catalase and methyl red positive and reduced nitrate and hydrolysed starch. The other isolates were Gram negative rods and tested positive for MethylRed Voges-Proskauer, catalase and indole, negative for citrate test, reduced nitrate and



Yellowish green pigment on Kings B medium

Creamish pigment on Nutrient Agar medium

Figure 1. Cultural characteristics of rhizobacterial isolates.

Table 1. Comparative growth of rhizobacteria on media containing ACC/ Ammonium sulphate as sole nitrogen source.

Rhizobacteria	OD_{600}		
	ACC (+)	ACC(-)	Ammonium sulphate
4-B	0.424	0.020	0.621
8-B	0.406	0.035	0.644
23-B	0.463	0.015	0.730
24-B	0.260	0.051	0.572
25-B	0.351	0.015	0.574
1-P	0.217	0.019	0.248
4-P	0.273	0.013	0.670
6-P	0.317	0.053	0.693
10-P	0.180	0.022	0.250
15-P	0.176	0.060	0.347

hydrolysed starch. On the basis of these tests, the isolates were tentatively assigned to genera *Bacillus* (B) and *Pseudomonas* (P).

Aminocyclopropane-1-carboxylic acid (ACC)-deaminase activity

Of the 47 rhizobacteria, 10 were able to utilize ACC as sole N-source, however, variation in efficacy to utilize ACC was observed (Table 1). The *Bacillus* isolates (23-B, 4-B, 8-B, 25-B and 24-B) showed highest growth in terms of O.D λ_{600}(0.463, 0.424, 0.406, 0.351 and 0.260) whereas Pseudomonads (6-P, 4-P, 1-P 10-P and 15-P) showed medium growth (0.317, 0.273, 0.217, 0.180 and 0.176). Several bacterial species belonging to different genera have been reported to exhibit variable ACC-deaminase activity.

Phosphate-solubilization

Out of 10 ACC-deaminase positive isolates, three isolates showed varying P-solubilizing index of 2.2 to 2.7, highest being with isolate 23-B followed by 6-P. Under liquid culture P-solubilized ranged from 14.6 to 21.6 mg/100 ml. The rhizobacteria 6-P was a potent P-solubilizer showing maximum P-solubilization on 6th day of incubation (21.6 mg/100 ml) followed by 8-B (20.2 mg/100 ml) and 23-B (17.9 mg/100 ml) (Table 2). Mahalakshmi and Reetha (2009) reported that 83.3% of *Pseudomonas* isolates were P-solubilizers.

Indole-3-acetic acid (IAA) production

Diverse soil microorganisms are known to produce IAA, which is an important hormone for plant growth, root initiation and elongation (Yasmin et al., 2009). All the rhizobacteria produced IAA, *Bacillus* (11.2-22.8 µg/ml) and *Pseudomonas* isolates (4.4-21.6 µg/ml), highest being 23-B (22.8 µg/ml) (Table 2). In the present study *Bacillus* spp were found to be strong IAA producers. These results are in accordance to Etesami et al. (2009) they reported significant difference in IAA production amongst the isolates.

Table 2. Comparative performance of rhizobacteriain terms of plant growth promoting traits.

Rhizobacteria	P-solubilization Index	IAA equivalents (µg/ml)		P-solubilization (mg/100 ml)	
		L-TRP (-)	L-TRP(+)	6th Day	9th Day
4-B	-	1.4	19.4	-	-
8-B	2.2	8.8	18.2	20.2	17.2
23-B	2.7	4.7	22.8	14.6	17.9
24-B	-	3.5	13.6	-	-
25-B	-	8.8	11.2	-	-
1-P	-	1.7	11.9	-	-
4-P	-	4.2	10.0	-	-
6-P	2.4	5.6	21.6	21.6	18.2
10-P	-	0.5	4.4	-	-
15-P	-	3.4	8.2	-	-

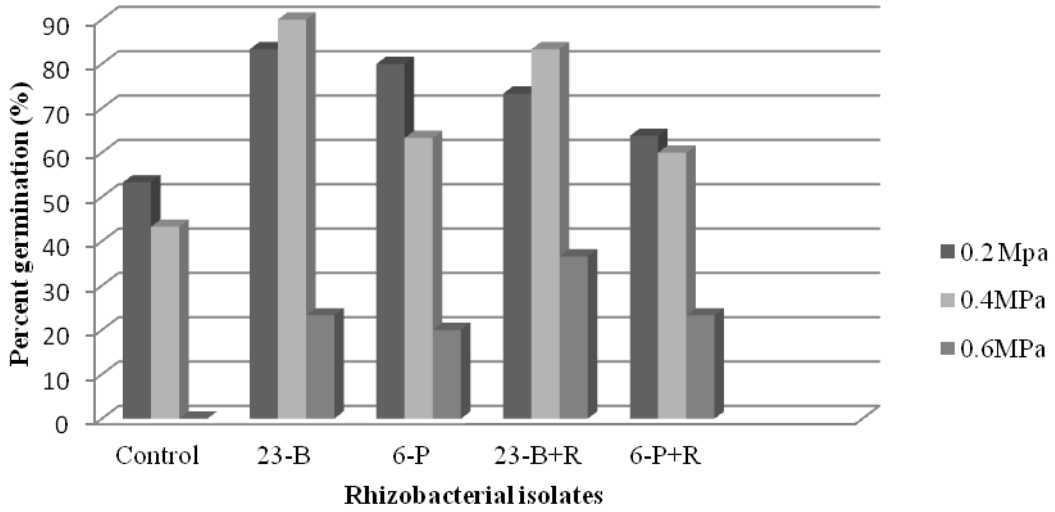


Figure 2. Effect of ACC-deaminase positive rhizobacteriaon germination of chickpea L-552 seeds under water stress.

Effect of aminocyclopropane-1-carboxylic acid (ACC)-deaminase positive rhizobacteria on germination and growth of chickpea L-552 under water stress

Germination of chickpea (L-552) seeds was affected by PEG induced water stress. At 0.2 MPa seeds inoculated with isolate 23-B showed maximum germination (83.3%) followed by 6-P (80%) as compared to uninoculated control (53%). Isolate 23-B in combination with *Mesorhizobium* showed better germination as compared to isolate 6-P. At 0.4 Mpa, isolate 23-B showed 90% germination followed by 23-B+R (83.3%), 6-P (63.3%) and 6-P+R (60%) over control (43%), whereas at 0.6 MPa, 23-B+R (36.6%) and 23-B (23.3%), exhibited germination, however, no germination was observed in control. From the data it is evident that isolate 23-B induced maximum water stress tolerance in chickpea L-552 as compared to isolate 6-P (Figure 2).

Root growth was also greatly affected by water stress at all the three levels (Table 3). At 0.2 and 0.4 MPa maximum increase in root elongation was recorded in seeds treated with PGPR 23-B (3.6 and 2.7 cm) whereas at 0.6 MPa, isolate 6-P+R (1.2 cm) was more effective. At 0.2 MPa application of isolate 23-B+R recorded root fresh weight of 70.3 mg/seedling followed by isolates 23-B, 6-P, 6-P+R (60, 57 and 52.7 mg/seedling), at 0.4 MPa isolate 23-B alone gave maximum root fresh weight (45 mg/seedling) but at 0.6 MPa isolate 23-B+R increased root fresh weight to maximum extent (18.3 mg/seedling). Shoot traits also exhibited significant decrease under stress, seeds showed no plumule emergence at 0.6 MPa stress. At 0.2 MPa, isolate 23-B+R showed maximum shoot length (1.2 cm) followed by 23-B (1 cm), at 0.4

Table 3. Effect of ACC-deaminase positive rhizobacteria on root and shoot traits of chickpea L-552 under water stress.

Rhizobacteria	PGP trait											
	Root length (cm)			Root fresh weight (mg/seedling)			Shoot length (cm)			Shoot fresh weight (mg/seedling)		
	Doses of PEG (MPa)											
	0.2	0.4	0.6	0.2	0.4	0.6	0.2	0.4	0.6	0.2	0.4	0.6
Control	2.1	1.6	-	28.3	27	-	0.53	-	-	15.6	-	-
23-B	3.6	2.7	0.6	60.0	45	12.7	1	0.6	-	22.7	25	-
23-B+R	2.1	2.3	0.8	70.3	36.3	18.3	1.2	0.5	-	24	21	-
6-P	2.7	1.9	0.6	57.0	30	16.7	-	-	-	-	-	-
6-P+R	2.1	1.6	1.2	52.7	22.3	13.3	-	-	-	-	-	-
Absolute control		5.9			100.0			1.3			52.7	
CD (P = 0.05)	1.7	1.9	1.3	34.6	25.5	13.2	0.48	0.52	-	28.1	26.6	-

MPa, 23-B showed maximum shoot length (0.6 cm) Followed by 23-B+R (0.5 cm). Shoot fresh weight was maximum at 0.2 MPa when inoculated with isolate 23-B+R, followed by 23-B (24 & 22.7 mg/seedling) as compared to control (15.6 mg/seedling). At 0.4 MPa isolate 23-B showed maximum shoot fresh weight (25 mg/seedling) (Figure 3). It is clear from the data that 23-B alone and along with *Mesorhizobium* exhibited profound effect on chickpea growth under water stress. These results are in corroboration with results of Mayak et al. (2004) who reported that ACC-deaminase PGPR *Achromobacter piechaudii* ARV8 significantly increased the fresh and dry weights of tomato and pepper seedlings exposed to transient water stress.

Effect of aminocyclopropane-1-carboxylic acid (ACC)-deaminase positive rhizobacteria on germination and growth of chickpea GPF-2 under water stress

Chickpea variety GPF-2 also showed significant reduction in plant growth under water stress conditions and germination was inhibited at osmotic potential (0.6 MPa) which is considered as threshold potential (Table 4). Only radicle emergence was noticed in GPF-2 variety whereas plumules failed to emerge at all the three water stress levels. Here too, at 0.2 MPa isolates 23-B and 23-B+R showed maximum germination (63.3%) and at 0.4 MPa 23-B+R showed maximum germination (56.6%) followed by isolate 23-B (40%) over uninoculated control (Figure 4). At 0.2 MPa and 0.4 MPa seed inoculation with 6-P+R gave maximum root elongation (3.0 and 3.1 cm) over control (1.8 cm). At 0.2 MPa isolate 23-B exhibited profound increase in root fresh weight whereas, at 0.4 MPa, 6-P+R showed higher root fresh weight ((53 &32 mg/seedling) as compared to uninoculated control (18.3 mg/seedling) (Figure 5). This study shows that water stress inhibited coleoptile emergence more than the radicle which is in accordance to results of Macar et al. (2008). The ACC-deaminase containing rhizobacteria probably reduced the endogenous ethylene levels in chickpea at early stage of development thus enhanced their growth and root elongation under stress. *Bacillus* isolate 23-B was found to be more effective in plant growth promotion probably due to its higher ACC-deaminase activity. Treatment with ACC deaminase-producing bacteria typically reduces ethylene levels by about 2-4 fold (Reed and Glick, 2005). It has been reported ACC deaminase-PGPR protects the plants against damage from drought, high salt and polyaromatic hydrocarbons (Mayak et al., 2004; Glick et al., 2007).

Proline accumulation

The proline content shows an increasing trend in both chickpea varieties under water stress which is in accordance to the result of Madhurendra (2009). Treatment with *Bacillus* isolate 23-B shows maximum proline accumulation (0.80 and 0.66 mg/g fresh weight radicle) in comparison to *Pseudomonas* isolate 6-P (0.32 and 0.31) in both chickpea varieties as compared to control and absolute control (Table 5). Co-inoculation of isolate 23-B with *Mesorhizobium* also gave similar results. These results are in corroboration with work of Singh (2004) who reported accumulation of proline in saline stress tolerant chickpea genotypes. Slow utilization of proline for protein synthesis during stress results in its accumulation. Proline is known to act as a compatible osmolyte, antioxidant and maintains cytosolic pH (Verbruggen and Hermans, 2008).

Conclusion

The present study indicates that co-inoculation of ACC-deaminase producing PGPR with *Mesorhizobium* significantly promoted growth of chickpea by positively influencing seed germination andother growth factors under

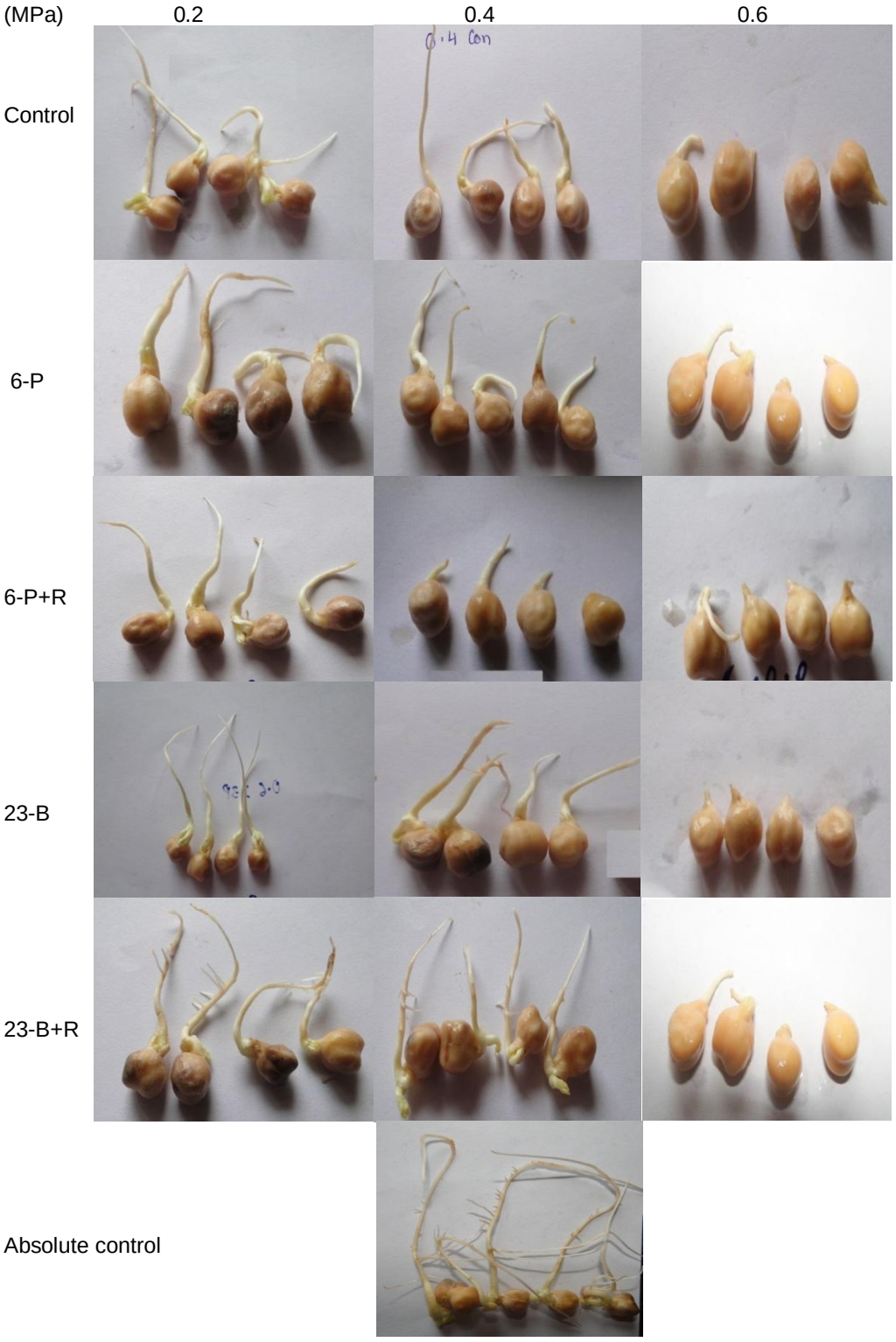


Figure 3. Effect of ACC-deaminase positive rhizobacteria on germination and growth of chickpea (L-552) under water stress

water stressed conditions, most probably through lowering of ethylene. The efficiency of application of such inoculants under field conditions would further elucidate their potential in stress amelioration. Screening efficient

Table 4. Effect of ACC-deaminase positive rhizobacteria on root traits of chickpea GPF-2 under water stress.

Rhizobacteria	PGP trait					
	Root length (cm)			Root fresh weight (mg/seedling)		
	Doses of PEG (MPa)					
	0.2	0.4	0.6	0.2	0.4	0.6
Control	1.8	1.5	-	38.7	18.3	-
23-B	2.7	1.7	-	53.0	20.0	-
23B+R	2.8	2.3	-	45.3	21.0	-
6-P	2.3	-	-	49.7	-	-
6-P+R	3.0	3.1	-	42.0	32.0	-
Absolute control		2.6			40.7	
CD (P = 0.05)	NS	1.8	-	NS	13.7	-

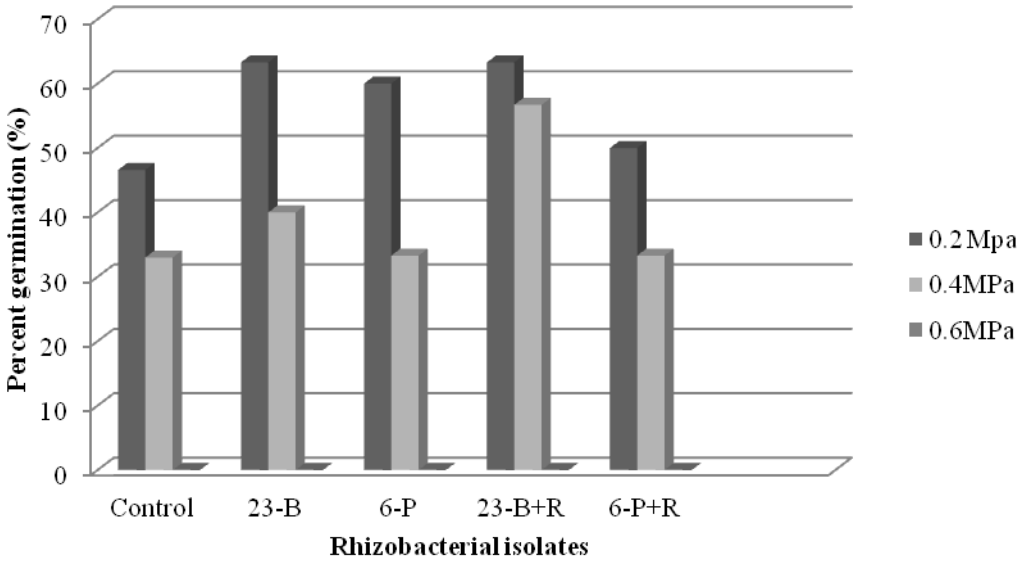


Figure 4. Effect of ACC-deaminase positive rhizobacteria on germination of chickpea GPF-2 seeds under water stress.

Table 5. Proline content in water stress induced radicles of chickpea.

Rhizobacteria	Proline content (mg/g fresh weight)	
	GPF-2	L-552
6-P	0.32	0.31
6-P+R	0.52	0.60
23-B	0.80	0.66
23-B+R	0.38	0.42
Control*	0.19	0.21
Absolute control**	0.29	0.24

Data recorded at 0.2 MPa PEG induced water stress. *Uninoculated seeds under water stress. **Uninoculated seeds under stress free conditions.

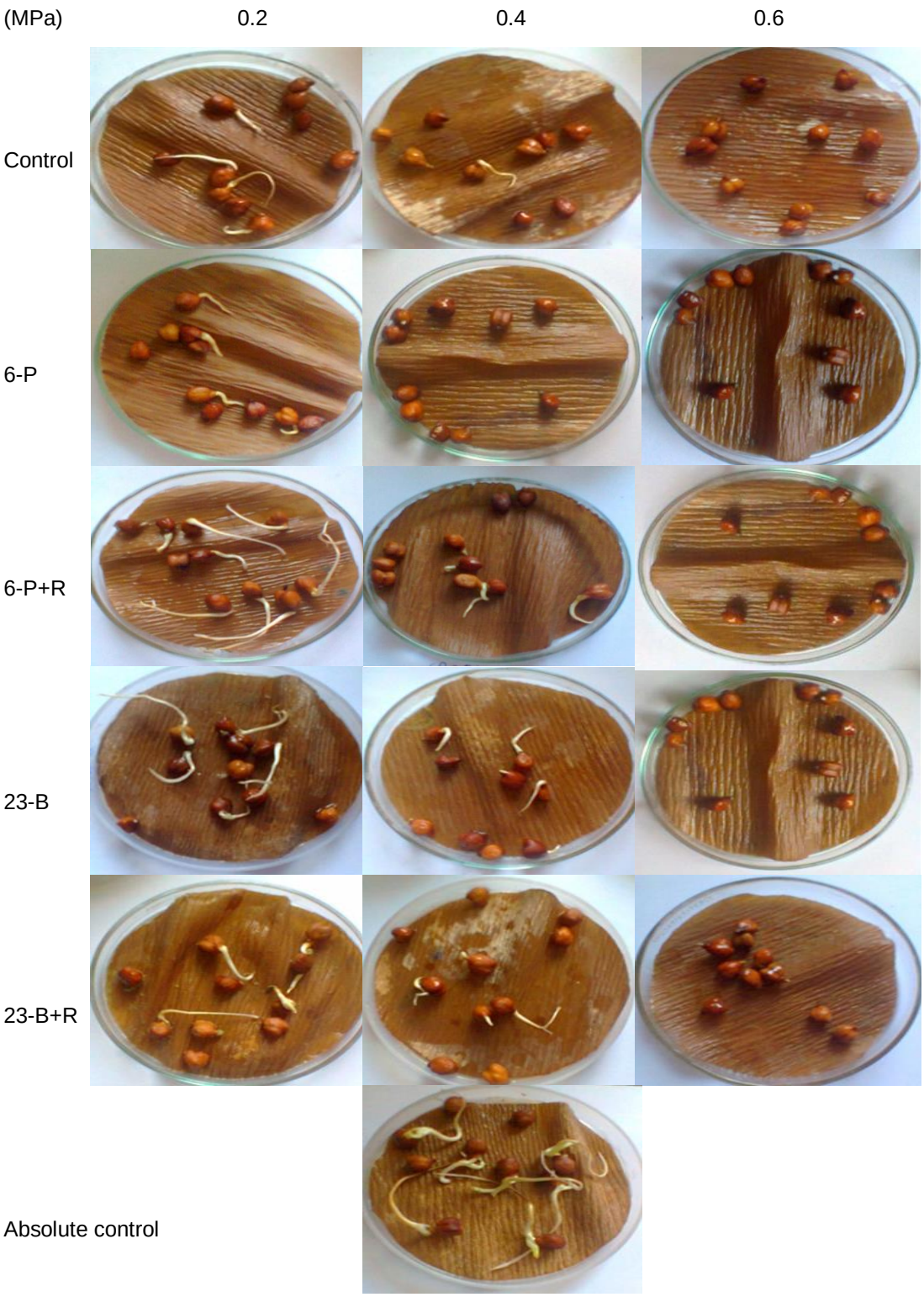


Figure 5. Effect of ACC-deaminase positive rhizobacteria on germination and growth of chickpea (GPF-2) under water stress.

PGPR's containing ACC-deaminase activity compatible with the environment and plant hostis thus a very useful approach to enhance the nodulation and growth in legumes under stress conditions.

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Full Length Research Paper

Effect of *Helicobacter pylori* infection on oxidative stresses in patients with chronic gastritis

Azita Navvabi[1], Mohammad Hassan Khadem Ansari[2]*, Nazila Navvabi[3], Sara Khadem Ansari[4] and Yousef Rasmi[2]

[1]Department of Microbiology, Zanjan Branch, Islamic Azad University, Zanjan, Iran.
[2]Department of Clinical Biochemistry, Urmia Medical School, Urmia, Iran.
[3]Department of Veterinary Medicine, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.
[4]Department of Medicine Tabriz Azad University, Tabriz, Iran.

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The *Helicobacter pylori* infection is currently endemic worldwide with high prevalence in developing regions. The infection causes chronic gastritis, gastric and gastric adenoid carcinoma. Infection by *H. pylori* may act as a risk factor for proteins, lipids and DNA damages. In this study, gastric biopsies were obtained in patients with a chronic gastric to investigate effects of *H. pylori* infection on oxidative stress in patients with chronic gastritis. Urease test and polymerase chain reaction (PCR) were performed on endoscopic gastric biopsy obtained from 150 subjects. The result showed that the presence of a 492-bp DNA fragment was indicative of a positive reaction, also fresh samples had a positive urease test for *H. pylori.* Malondialdehyde (MDA) in patients with gastritis as a result of *H. pylori* were significantly increased as compared to the control groups ($p<0.05$). Mean ± SE of reduced glutathione (GSH) and total antioxidant capacity (TAC) in patients were decreased significantly ($p<0.05$). The result shows mean ± SE of oxidized glutathione (GSSG) in patients showed significant increased as compared to the control groups. These finding show that *H. pylori* infection can cause oxidative stress disorders that may act as risk factors.

Key words: Malondialdehyde, reduced glutathione, oxidized glutathione, total antioxidant capacity, *Helicobacter pylori*.

INTRODUCTION

Helicobacter pylorus is one of the most infectious human pathogens, as over half of the world's population is infected with this Gram-negative bacteria (Kusters et al., 2006). *H. pylori* possesses an unusual characteristic of urea hydrolysis, forming carbon dioxide and ammonia (Xia et al., 1994). The major etiologic association of chronic gastritis is chronic infection by *H. pylori* (Adisa et al., 2011).

Environmental and genetic factors appear to be important in the progression of *H. pylori* on initiated gastritis to more serious outcomes (Brown, 2000).The radicals also promote mucosal damage by causing degradation of the epithelial basement membrane components, complete alteration of the cell metabolism and DNA damage (Demir et al., 2003). The body possesses an antioxidant defense system that removes peroxides, free radicals and superoxide generated within the mucosa. Reduced glutathione plays an important role against tissue oxidative damage, its depletion results in the accumulation of free radicals which initiate bio-

*Corresponding author. E-mail: mhansari1@hotmail.com. Tel: +989141415879.

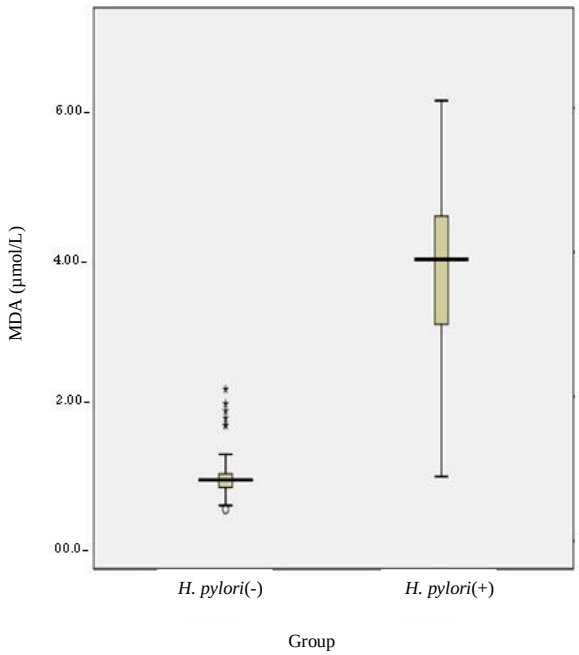

Figure 1. Malondialdehyde (MDA) in gastritis patients with and without *H. pylori* infection.

chemical damage by covalent binding to macromolecules and by lipid peroxidation (Santra et al., 2000). When antioxidant defenses are weakened, body cells and tissues become more prone to develop dysfunction (Kusano, 2008) then, the maintenance of adequate antioxidant levels is essential to prevent or even manage a great number of disease conditions (Kusano, 2008).

On the other hand, the use of total antioxidant capacity test, is as a biomarker of disease in biochemistry, medicine, food and nutritional sciences (Kusano, 2008). The changes of parameters such as malondialdehyde (MDA), reduced glutathione (GSH), oxidized glutathione (GSSG) and total antioxidant capacity (TAC) are a causative agent for chronic and acute gastritis and the study in this field is attractive for scientists.

The aim of this study was to investigate effects of *H. pylori* infection on MDA, GSH, GSSG and TAC in patients with chronic gastritis. This is a new subject of study in *H. pylori* infection in antrum of gastritis and oxidant and anti oxidant status.

MATERIALS AND METHODS

Endoscopic gastric biopsy were obtained from one hundred and fifty patients (males/females) aged between 30 to 50 years old with gastritis resulting from *H. pylori* (Sacco et al., 2007). Fraction of biopsy specimens 3 mm maintained from antrum was placed in 0.9% NaCl for PCR test. At the time of biopsy, five milliliters of blood were drawn from each of the patients and placed on ice. Serum was separated in refrigerated centrifuge at 6000 *g* and kept at -80°C until they were tested.

Urease test

One fraction from the biopsy specimen was used for urease testing for *H. pylori*. The change in colour of the broth from pale yellow to deep pink was taken as a positive test. On urease positive samples (Berry,2006), PCR test were carried out .

Sixty eight gastritis patients were *H. pylori* positive by PCR and urease positive were chosen as patient groups, 68 gastritis patients without *H. pylori* infection were chosen as a control groups

Helicobacter pylori PCR detection

H. pylori PCR detection was carried out by DNA extraction using CinnaGen DNP (#DN8116C) and amplification of samples were carried out by CinnaGen *H.pylori* PCR detection kit (CinnaGen Molecular Biology and Diagnostic,Tehran,Iran), and then electrophoresis was performed by using 100 bp ladder as a marker in 1.5% agarose gel, and positive samples show a single band at 492 bp DNA fragment.

Measurement of oxidized and reduced glutathione GSSG/GSH, malondialdehyde, total antioxidant capacity

GSSG/GSH was assayed using instruction for factory kit (Oxford Bio Medical Research USA). For measurement of GSSG, 30 µL of scavenger from GSSG kit was added to 100 µl of serum and then all serum samples were kept at -80°C until they were tested. MDA levels in samples were spectrophotometrically determined (Khanzode et al., 2003).

Total antioxidant capacity test was performed by randox kit (Randox laboratories, UK) and samples were measured automatically with BT-3000 (Biotechnical Instruments, Italy). The amount of total antioxidant was reported (mmol/L). Statistical calculations were done by SPSS 16 and P value less than 0. 05 were accepted as significant.

RESULTS

In this study, MDA, GSH, GSSG and TAC concentrations in serum samples from chronic gastritis patients with *H. pylori* and without *H. pylori* infection were studied. Results show that MDA, GSH and GSSG and TAC levels in patients were different from that of the controls.

MDA levels and mean values with standard errors in patient groups and control groups were 3.75±0.15 and 0.92±0.04 µmol/L, respectively ($p<0.05$) (Figure 1). The GSH values of the patient groups were 0.59±0.03 µmol and the control group were 2.14±0.08 µmol, statistical comparisons revealed significant differences between the two groups ($p<0.05$), results indicate a decrease in glutathione levels in the patient group than the control group (Figure 2).

Oxidized glutathione in patient groups and control groups were 0.26±0.01 and 0.05±0.03 µmol, respectively. Levels of GSSG were significantly increased ($p <0.05$) (Figure 3). The results of the TAC show that in the patient groups it was 0.92±0.04 mmol/L and the control groups it was 1.70±0.06 mmol/L ($p <0.05$) (Figure 4). The results suggest a correlation between GSSG, GSH and GSSG with MDA present in patient groups.

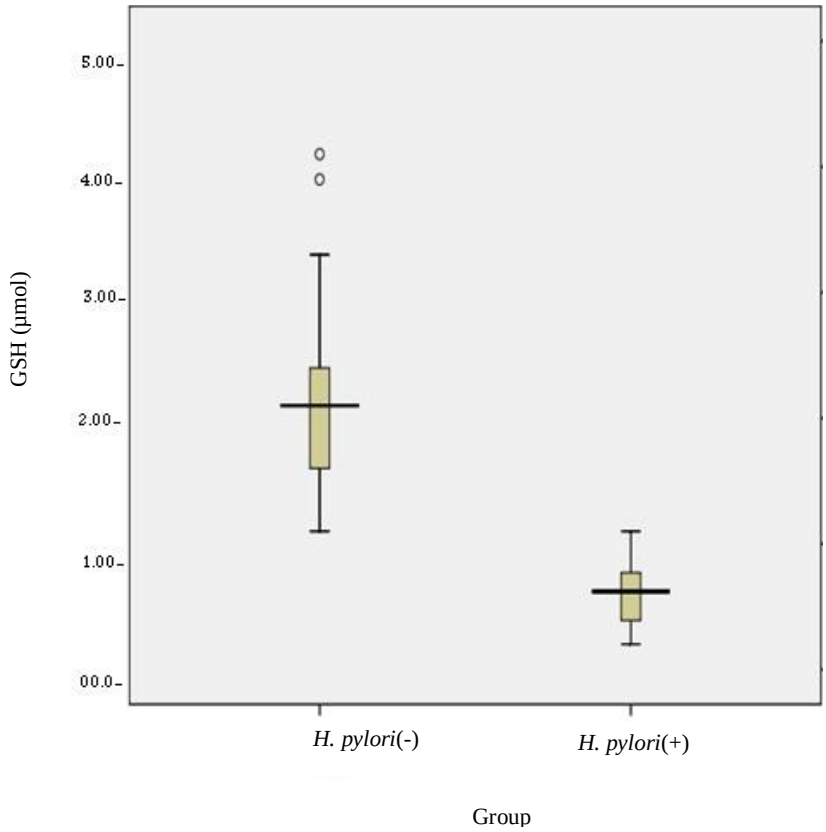

Figure 2. Reduced glutathione (GSH) in gastritis patients with and without *H. pylori* infection.

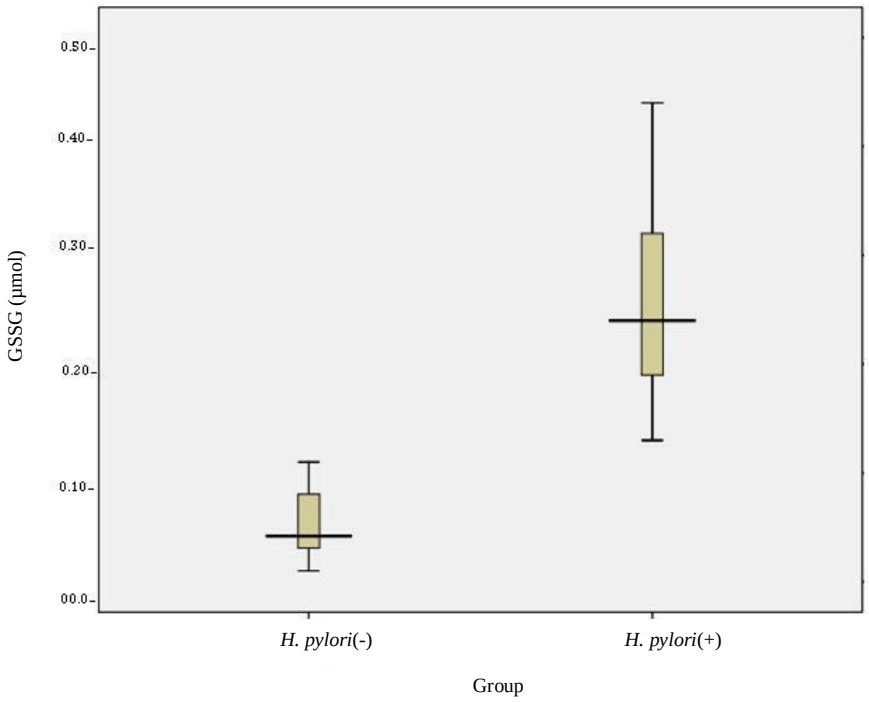

Figure 3. Oxidized glutathione (GSSG) in gastritis patients with and without *H. pylori* infection.

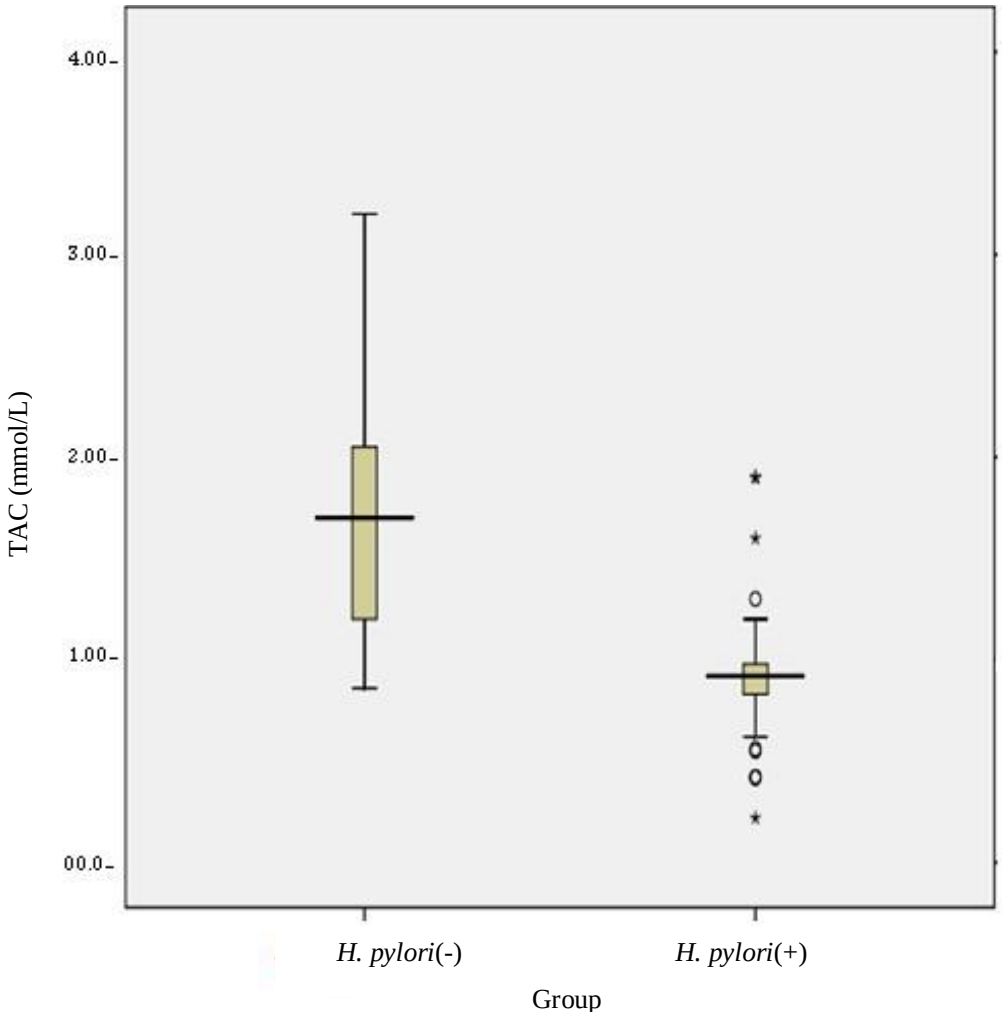

Figure 4. Total antioxidant capacity (TAC) in gastritis patients with *H. pylori* infection (case) and gastritis patients without *H. pylori* infection (control).

DISCUSSION

H. pylori is one of the most infectious human pathogens, as over half of the world's population is infected with this Gram-negative bacteria (Kusters et al., 2006).The prevalence of *H. pylori* infection appears to be higher in developing countries (Adisa et al., 2011). Reactive oxygen species (ROS) produced by this bacteria may be one of the crucial factors whereby oxidative stress can play a role in the pathogenesis of ulcer disease (Arend et al., 2005).

In the present study, *H. pylori* effects on oxidative stress including MDA, GSH and GSSG and TAC were tested. Our findings with the aim of studying *H. pylori* effects on oxidative stress in chronic gastritis patients showed that the amount of malondialdehyde and oxidized glutathione were increased in patients, levels of total antioxidant and reduced glutathione were decreased in these patients. Significant increase in serum superoxide dismutase and serum malondialdehyde was observed in *H. pylori* gastritis and gastric cancer patients as compared to control subjects (Khanzode et al., 2003). Our findings are concordant with these observations. It is known that radical scavengers play a significant role in protecting membranes from oxidative damage (Demir et al., 2003).

Depletion of gastric mucosal GSH may result in the accumulation of free radicals that can initiate membrane damage by lipid peroxidation (Demir et al., 2003). A deficiency of GSH puts the cell at risk for oxidative damage (Demir et al., 2003). *H. pylori* directly decrease cellular glutathione. A decreased level of GSH has been detected in the gastric mucosa of patients with *H. pylori* infection (Beil et al., 2000). In *H. pylori*-positive subjects, the sera values of total antioxidative status were significantly lower as compared to *H. pylori* m negative while the ratio between oxidized and reduced glutathione decreased in *H. pylori*-positive subjects (Hutt et al.,

2008). The results are in agreement with our findings.

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Full Length Research Paper

Comparison of the conventional technique and 16S rDNA gene sequencing method in identification of clinical and hospital environmental isolates in Morocco

Meriem El Bakkali[1,2], Imane Chaoui[2], Mimoune Zouhdi[3], Marouane Melloul[4], Abdelhay Arakrak[1], Elmostafa Elfahime[4], Mohammed El Mzibri[2] and Amin Laglaoui[1]*

[1]Equipe de Recherche Biotechnologies et Génie des Biomolécules (ERBGB) FST-Tanger, Morocco.
[2]Unité de Biologie et. Recherche Médicale CNESTEN-Rabat, Morocco.
[3]Service de microbiologie de l'hôpital Ibn Sina de Rabat, Morocco.
[4]Unités d'Appui Techniques à la Recherche Scientifique, CNRST-Rabat, Morocco.

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Early and effective diagnosis of infectious diseases of bacterial origin is a critical key in the management of public health. In hospitals, accurate identification of bacterial isolates is an essential task for the microbiological control. Small subunit ribosomal RNA gene (16S rDNA) sequence analysis is usually used for the identification and classification of bacteria. To evaluate the accuracy of 16S rDNA gene sequencing in the identification of bacteria, 58 clinical and hospital environmental isolates were identified by both conventional and molecular techniques. The comparison between the conventional identification and the 16S rDNA gene sequence identifications showed that the genus identification overlapped for both methodologies in 93.1% of the cases and the species identification in 60.34% of the cases, 16S rDNA gene sequencing had a high percent accuracy as compared to the conventional methods. The obtained results suggest that combination of conventional methods and 16S rDNA gene sequencing provide a more accurate identification of clinical and environmental bacteria to enhance the human health management.

Key words: Clinical bacteria, conventional identification, 16S ribosomal RNA, DNA sequencing.

INTRODUCTION

During the last decades, nosocomial infection was Registered with constant increase worldwide. A bleaker picture emerged with the discovery of extreme form of drug resistance, especially for new generation drugs that pose a great threat to the success of patients' treatment (Podschum and Ulmann, 1998). Hospital environments are responsible of the dissemination of micro-organisms for different distances and progressive contamination of various supports, including surfaces (Bonten et al., 1996; Boyce et al., 1997) air and water (Curtis, 2008), and constitute therefore a possible source of nosocomial infections (Danforth et al., 1987; Maki et al., 1982; Huang et al., 2006; Sexton et al., 2006). Thus, accurate identification of bacterial isolates from the hospital environment is an essential task for the microbiological control.

In Morocco, like other developing countries, bacterial identification is still based on the use of conventional techniques, including Gram staining, colonies morphologies, growth requirements and enzymatic and/or metabolic activities. This phenotypic approach presents some

*Corresponding author. E-mail: laglaouiamin@yahoo.fr.

inherent problems: they are time consuming, fail to identify some bacteria because of the variability of characteristics generated with stress or evolution, and the test results may be based on an individual and subjective interpretation (Stager and Davis, 1992; Ochman et al., 2005). These techniques cannot distinguish between strains belonging to the same species. Moreover, the corresponding database may not yet include newly described species or unusual microorganisms (Stager and Davis, 1992).

Currently, molecular approach based on the 16S rDNA gene sequencing is used in different clinical laboratories for routine identifications, especially for slow-growing, unusual or fastidious bacteria, but also for bacteria that are poorly differentiated by conventional methods (Drancourt et al., 2000; Kupila et al., 2003; Tsai et al., 2004; Petti et al., 2005). Identification based on the 16S rDNA sequence is of interest because ribosomal small subunit exists universally among bacteria and includes regions with species-specific variability, which makes it possible to identify bacteria to the genus or specie levels by comparison with databases in the public domain (Vandamme et al., 1996). A direct comparison of rDNA sequences is probably the most powerful tool for the tracing of phylogenetic relationships between bacteria from various sources, such as environmental or clinical specimens (Drancourt et al., 2000) and their identification (Stackebrandt and Goodfellow, 1991).

16S rDNA sequencing could be performed on DNA from bacterial cultures but also directly from specimens to study the diversity of microorganisms without culturing (Lane et al., 1985; Gray and Herwig, 1996; Gill et al., 2006; Rajendhran and Gunasekaran, 2008). van der Heijden et al. (2000) have clearly demonstrated the interest of using 16S rDNA sequencing in the identification of novel organisms of unknown or poorly defined pathogenicity from patient samples.

The efficacy of bacterial identification by 16S rDNA sequencing and conventional techniques was already evaluated. Reported results clearly demonstrate that the 16S rDNA sequences is more efficient than classical phenotypic methods for the identification of atypical bacteria of clinical origin (Morgan et al., 2009) and from freshly isolated from a natural environment (Boivin-Jahns et al., 1995).

Moreover, the efficacy of genotypic identification using 16S rDNA sequencing was clearly demonstrated in the identification of microorganisms misidentified by conventional methods (Petti et al., 2005; Cherkaoui et al., 2009).

The great potential of the molecular approach has been reported for Gram-positive rods and coryneiform bacterial identification (Bosshard et al., 2003; Tang et al., 2000), for Gram-positive, catalase-negative cocci (Bosshard et al., 2004) and for Gram-negative rods (Tang et al., 1998; Coenye et al., 2002; Ferroni et al., 2002).

Subsequent studies have supported the use of broad range 16S rDNA PCR as a valuable adjunct for increasing diagnostic sensitivity of some bacterial diseases (Lu et al., 2000; Pandit et al., 2005; Xu et al., 2005), particularly in culture-negative cases (Fenollar et al., 2006).

Moreover, the 16S rDNA identification is the only effective diagnostic method that could provide an etiological diagnosis when the patient is receiving antibiotics, or when the causative agent is a fastidious bacterium (Kupila et al., 2003; Tsai et al., 2004). Thus, poorly described, rarely isolated or phenotypically aberrant strains could be better identified by 16S rDNA gene sequencing.

Moreover, this technique could lead to the discovery and description of novel pathogens and facilitate the identification of non-cultured bacteria.

Currently, 16S rDNA gene sequence analysis is more expensive than most traditional identification methods, for routine identifications. However, for difficult organisms, multiple identification methods often must be used, which increases the cost (Patel et al., 2000; Patel, 2001; Wilck et al., 2001; Cook et al., 2003; Hall et al., 2003; Voldstedlund et al., 2008). Such exhaustive phenotypic testing potentially delays turnaround time without the added benefit of accuracy.

Generally, bacterial identification is based on the full length 16S rDNA gene sequencing (1500 bp), but several studies described the use of the initial 500 bp sequence which provides a sufficient discrimination between strains because this region shows a high genetic diversity (Kattar et al., 2001).

Thus, this study was planned to compare bacterial identification by 16S rDNA sequencing and the conventional techniques, using samples from the hospital environment in order to assess the use of this molecular approach for identifying bacteria in a routine clinical microbiology screening.

MATERIALS AND METHODS

Study design

The comparative study was conducted on 58 bacterial strains, isolated from a hospital survey during 2010. Isolates were collected from patients and the hospital environment including hands of hospital personnel and admitted patients, and various surfaces and locations. Isolates were identified by both conventional technique and molecular approach based on 16S rDNA ID. The accuracy of 16S rDNA ID was evaluated by comparing results of 16S rDNA gene sequencing to the results of the conventional technique considered as gold standard approach.

Conventional identification

Spits were inoculated onto the Chocolat enriched or bacitracin agar in 5% CO_2, and for *Neisseria* sp., the 5% sheep blood agar was used. Blood was injected into two or more "blood bottles" with specific media for aerobic and anaerobic organisms and sub cultured onto 5% sheep blood agar for *Streptococcus pneumoniae*, Chapman agar plates for Gram-positive *Staphylococcus,* Desoxycholate citrate lactose agar (DCL agar) plates for Gram-negative bacteria and Chocolate agar for exigent bacteria.

Cerebrospinal fluid was inoculated onto Chocolat enriched/ bacitracin agar in 5% CO_2. The urine specimens were inoculated

onto phosphate buffered saline agar, Cystine Lactose Electrolyte Deficient agar and Mac Conkey agar. The other clinical specimens were inoculated onto DCL agar, 5% sheep blood agar, selenite broth for *Salmonella* species, Chocolat enriched/bacitracin agar in 5% CO_2 for *Neisseria* sp. and *Haemophilus* sp.

Swabs were vortexed and subcultured on Chapman agar plates for Gram-positive bacteria, DCL agar plates for Gram-negative bacteria and Chocolate agar for exigent bacteria. The plates were incubated for 18 to 24 h at 37°C and visible colonies were further subcultured and incubated for 24 h at 37°C.

Isolation and identification of microorganisms were done according to standard procedures. Bacteria were identified by examination of colonial morphology, haemolytic characteristics on appropriate agar media, Gram staining, rapid tests (catalase, oxidase, coagulase/Dnase, optochin disc, bile solubility, spot indole, latex agglutination), and classic and API galleries (BioMérieux, France) (Baron and Finglod, 1996).

Bacterial DNA extraction

Bacterial DNA was extracted using the Sigma's GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, France), according to manufacturer instructions. Briefly, 1.5 mL of bacterial broth culture was pelleted at 12,000 to 16,000 ×g for 2 min; cells were resuspended in 180 µL lysis solution A for Gram-negative bacteria or in 200 µL of lysozyme (200 units/ml) for Gram-positive bacteria. Then, 20 µL of Proteinase K was added to the cell suspension. After incubation at 55°C for 30 min, 200 µL of lysis solution C was added to the suspension. The suspended cells were then incubated at 55°C for 10 min. DNA was purified using GenElute Miniprep Binding Columns (Sigma-Aldrich, France). DNA is then eluted in sterile distilled water and stored at -20°C until use.

16S rDNA gene sequencing

The 16S rDNA gene was amplified using primers fD1 (5-AGA GTT TGA TCC TGG CTC AG-3′) and rP2 (5′-AAG GAG GTG ATC CAG CC-3′), as described by Weisburg et al. (1991). PCR was performed using 2.5 µl of 10X buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.4 µM of each primer, 1 U of Platinum Taq Polymerase (Invitrogen) and 5 µl (30 ng/µl) of template DNA in a 25 µl reaction volume under the following conditions : 4 min at 96°C (initial denaturation), 35 cycles of 10 s at 96°C (denaturation), 40 s at 52°C (annealing), 2 min at 72°C (extension), and one final step of 4 min at 72°C (extension cycle) employing the PCR thermocycler "Verity" (Applied Biosystems). The amplified fragments were electrophoresed on 1% agarose gels and detected using ethidium bromide along with molecular weight markers. The PCR products were purified using EXOSAP-IT (USB, USA) and bidirectionally sequenced on an ABI 3130xl automated sequencer (Applied Biosystems) using BigDye Terminator version 3.1 Kits with the same primers as those used for the amplification. Analysis of electrophoregramm was done with the sequencing Analysis Software version 5.3.1 (Applied Biosystems). The consensus sequences were edited and compared with published sequences available in GenBank, using Blast tool of the NCBI. The criterion used to identify an isolate to the genus or species level, was suggested by several researches: 97 and 99% identity in 16S rRNA gene sequence to identify an organism to the genus and the species level respectively (Drancourt et al., 2000; Stackebrandt et al., 2002; Bosshard et al., 2003; Harris and Hartley, 2003; Clarridge, 2004; Janda and Abbott, 2002).

Comparative analysis

Results for each sample were compiled in a local database. For each isolate, comparative analysis was made between the conventional method and 16S rDNA gene sequencing, at genus and species levels, to genus level only, or no correlation. The percent accuracy for 16S rDNA gene sequencing method was calculated using the number of identifications out of the total number of samples tested.

RESULTS

16S ribosomal DNA gene sequencing

Bacterial strains collected from the Ibn Sina Hospital fell into one of the two designated categories of this study: Gram-negative rods and Gram-positive cocci. Conventional identification showed that 84.5% of the isolates are Gram-negative bacteria (49/58) and the main isolates were *Klebsiella pneumonia* (18 isolates), *Escherichia coli* (7 isolates) and *Acinetobacter baumannii* (6 isolates). The conventional method allowed also the identification of 10 *Pseudomonas*, including 4 *Pseudomonas aeruginosa*; 8 *Staphylococcus*, including 2 *Staphylococcus warneri*, 1 *Staphylococcus cohnii*, 1 *Staphylococcus sciuri*, 1 *Staphylococcus aureus* and 2 *Staphylococcus haemolyticus*; 6 *Enterobacter*; 2 *Proteus*, including 1 *Proteus mirabilis*; and 1 *Streptococcus*.

The correlation of 16S rDNA gene sequencing to conventional method was evaluated to determine percent accuracy of 16S rDNA gene sequencing for each level of identification (genus and species, genus, and no correlation). In this study, the comparison between the conventional identification and the16S rDNA gene sequence identifications showed that the genus identification overlapped for both methodologies in 93.1% (54/58) of the cases and the species identification in 60.34% of the cases (35/58).

There was no correlation between conventional method and 16S rDNA gene sequencing for 6.90% (4/58) of the study isolates.

Concordance at the genus and specie levels

The concordance at genus and specie levels was obtained for 35 strains (Table 1). Among them, 34 were Gram-negative bacteria and 1 was Gram-positive bacteria. For Gram-negative bacilli, Gram stain morphology and a manual biochemical profile appeared most consistent with identification as a *K. pneumonia* (17 isolates)*, E. coli* (6 isolates)*, P. aeruginosa* (4 isolates)*, Acinetobacter baumannii* (6 isolates) and *P. mirabilis* (1 isolates).

The only one Gram-positive cocci, giving a concordance at genus and specie levels, belonged to the *S. aureus* specie.

Concordance at the genus level

A total of 19 isolates, including 12 Gram-negative bacilli and 7 Gram-positive cocci, gave a concordance only at genus level (Table 2). Indeed, for Gram-negative bacteria, conventional identification was limited to genus;

Table 1. Comparison of conventional and 16S rRNA sequencing identification methods for bacteria/genus and specie levels.

	Strain	Identification by conventional methods	Using 16S rRNA gene sequencing (EzTaxon)
	2	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. rhinoscleromatis
	4	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. ozaenae
	15U	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. rhinoscleromatis
	36	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. ozaenae
	46	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. rhinoscleromatis
	51	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. rhinoscleromatis
	52	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. rhinoscleromatis
	55	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. rhinoscleromatis
	59	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. rhinoscleromatis
	60	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. ozaenae
	61	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. rhinoscleromatis
	62	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. ozaenae
	64U	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. pneumoniae
	67	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. rhinoscleromatis
	69	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. ozaenae
	73	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. ozaenae
Gram -	83	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* subsp. ozaenae
	6	*Escherichia coli*	*Escherichia coli* strain U 5/41
	32	*Escherichia coli*	*Escherichia coli* strain U 5/41
	44E	*Escherichia coli*	*Escherichia coli* KCTC 2441
	72	*Escherichia coli*	*Escherichia coli* strain U 5/41
	82	*Escherichia coli*	*Escherichia coli* KCTC 2441
	120	*Escherichia coli*	*Escherichia coli* KCTC 2441
	48	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* LMG 1242
	71	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* LMG 1242
	81	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* LMG 1242
	109P	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa* LMG 1242
	40E	*Acinetobacter baumannii*	*Acinetobacter baumannii*
	49	*Acinetobacter baumannii*	*Acinetobacter baumannii*
	50	*Acinetobacter baumannii*	*Acinetobacter baumannii*
	53U	*Acinetobacter baumannii*	*Acinetobacter baumannii*
	U56	*Acinetobacter baumannii*	*Acinetobacter baumannii*
	100U	*Acinetobacter baumannii*	*Acinetobacter baumannii*
	113	*Proteus mirabilis*	*Proteus mirabilis*
Gram +	43E	*Staphylococcus aureus*	*Staphylococcus aureus* subsp. aureus Mu50

A total of 34 Gram- and 1 Gram+ bacteria isolated from patients or hospital' environment have shown a concordance of identification at genus and specie levels by both conventional and 16S rDNA gene sequencing methods.

Pseudomonas or *Enterobacter*. Molecular identification based on 16S rDNA sequencing allowed the identification of two distinct species among *Pseudomonas* isolates, *P. aeruginosa* (5 isolates) and *P. Moorei* (1 isolate), and two distinct species among Enterobacter isolates; *E. cancerogenus* (4 isolates) and *E. hormaechei* (2 isolates).

Conventional identification showed that all Gram-positive bacteria reported in Table 2 belonged to *S. aureus* specie (7 isolates). However, the molecular approach revealed that there are 3 *S. haemolyticus*, 2 *S. warneri*, 1 *S. cohnii* subsp. Urealyticus and 1 *S. sciuri* subsp. Sciuri.

Absence of concordance

Table 3 summarises results of 4 isolates (3 Gram-negative and 1 Gram-positive bacteria) giving no concordance between conventional identification and molecular characterisation. The 3 Gram-negative isolates were identified

Table 2. Comparison of conventional and 16S rRNA sequencing identification methods for bacteria/genus level.

	Strain	Identification by conventional methods	Using 16S rRNA gene sequencing
Gram -	65	*Pseudomonas*	*Pseudomonas aeruginosa* LMG 1242
	70	*Pseudomonas*	*Pseudomonas aeruginosa* LMG 1242
	74	*Pseudomonas*	*Pseudomonas moorei* RW10
	77	*Pseudomonas*	*Pseudomonas aeruginosa* LMG 1242
	78	*Pseudomonas*	*Pseudomonas aeruginosa* LMG 1242
	79	*Pseudomonas*	*Pseudomonas aeruginosa* LMG 1242
	39	*Enterobacter*	*Enterobacter cancerogenus* LMG 2693
	53	*Enterobacter*	*Enterobacter cancerogenus* LMG 2693
	54	*Enterobacter*	*Enterobacter cancerogenus* LMG 2693
	64	*Enterobacter*	*Enterobacter cancerogenus* LMG 2693
	48E	*Enterobacter*	*Enterobacter hormaechei*
	55U	*Enterobacter*	*Enterobacter hormaechei*
Gram +	168P	*Staphylococcus aureus*	*Staphylococcus haemolyticus*
	5	*Staphylococcus aureus*	*Staphylococcus warneri* ATCC 27836
	80	*Staphylococcus aureus*	*Staphylococcus warneri* ATCC 27836
	33	*Staphylococcus aureus*	*Staphylococcus cohnii* subsp. urealyticus
	58	*Staphylococcus aureus*	*Staphylococcus sciuri* subsp. sciuri
	56	*Staphylococcus aureus*	*Staphylococcus haemolyticus*
	57	*Staphylococcus aureus*	*Staphylococcus haemolyticus*

A total of 12 Gram- and 7 Gram+ bacteria isolated from patients or hospital environment have shown a concordance of identification at genus level only by both conventional and 16S rDNA gene sequencing methods.

Table 3. Comparison of conventional and 16S rRNA sequencing identification methods for bacteria/absence of concordance.

	Strain	Identification by conventional methods	Using 16S rDNA gene sequencing
	55E	*K. Pneumoniae*	*Escherichia coli* O157
Gram -	63	*Proteus*	*Delftia tsuruhatensis* T7
	63U	*E. coli*	*Klebsiella pneumoniae* subsp. ozaenae
Gram +	28	*Streptococcus*	*Aerococcus urinaeequi*

A total of 3 Gram- and 1 Gram+ bacteria isolated from patients or hospital environment have shown no concordance of identification by conventional and 16S rDNA gene sequencing methods.

by the conventional technique as *K. pneumoniae, E. coli* and *Proteus*, whereas the molecular approach revealed a misidentification of these isolates at genus level identifying them respectively as *E. coli, K. pneumonia* and *Delftia tsuruhatensis*.

Moreover, a strong discordance was obtained with the Gram-positive bacteria which was identified as *Streptococcus* by conventional technique but identified as *Aerococcus urinaeequi* by 16S rDNA sequencing approach.

These results have demonstrated that the sequencing method allowed in several cases the possible identification of subspecies and strains of bacteria while the conventional methods failed to do so.

DISCUSSION

Worldwide, molecular approaches have emerged in clinical microbiology practices. Their high sensitivity, specificity and the short time required to perform the procedure explain the great interest given to these techniques in diagnostic laboratories. Rapid and accurate identification of bacterial isolates is a fundamental task in clinical microbiology, and provides insights into etiologies of infectious disease (Clarridge, 2004; Woo et al., 2008) and appropriate antibiotic treatment (Harris et al., 2002). Although conventional phenotypic methods are relatively inexpensive, easy to perform without the need for specialized instrumentation and allow identification of most com-

monly encountered bacteria, they fail to identify some special groups of bacteria, rare bacteria or bacteria with ambiguous profiles. Indeed, mistakes in identifying rarely encountered or phenotypically aberrant isolates are probably quite common in clinical microbiology laboratories. Sometimes, it is even difficult to know whether a bacterium has been incorrectly identified. Moreover, phenoltypic methods rely on the availability of pure culture and are dependent on subsequent growth characteristics and biochemical profiling.

Boivin-Jahns et al. (1995) have reported that the occurrences of misidentification of bacteria are very much decreased by using the 16S rDNA sequencing method.

16S rDNA sequencing represents a universal technology that, theoretically, provides solutions to these problems, yielding unambiguous data, even for unusual and slow-growing isolates, often within 48 h (Relman et al., 1990; Patel et al., 2000; Drancourt et al., 2004; Woo et al., 2008; Morgan et al., 2009), which are reproducible among laboratories (Kolbert and Persing, 1999; Drancourt et al., 2004). 16S rDNA sequencing is considered as a reference method for bacterial taxonomy and identification. It has been utilized by several researchers to identify environmental and clinical isolates (Stackebrandt et al., 1993; Drancourt et al., 2000; Clarridge et al., 2001; Clarridge and Zhang, 2002; Bosshard et al., 2003; Song et al., 2003; Drancourt et al., 2004; Drancourt and Raoult, 2005)

On the other hand, 16S rDNA identification is of great interest in determining the appropriate treatment of some isolates that are considered environmental contaminants (Miller and Rhoden, 1991; Drancourt et al., 2004) but can cause opportunistic infections in immunocompromised patients (Morgan et al., 2009, Sontakke et al., 2009), because they are rarely associated with human infection and considered clinically insignificant (Clarridge, 2004). Unlike phenotypic identification, which can be affected by the presence or absence of non-housekeeping genes or by variability in expression of characters, 16S rDNA sequencing provides precise identification of isolates with atypical phenotypic characteristics. In fact, the major advantage of 16S rDNA sequencing is the presence of the 16S rDNA gene in all bacteria; it provides high accuracy for identification of any bacterial organism, reliability and reproducibility (Kolbert and Persing, 1999; Drancourt et al., 2004).

In this study, results clearly demonstrate the feasibility of routinely used method in a microbiological laboratory, allowing the rapid and accurate identification of these pathogens, which is important in clinical and public health interventions. Most of the isolates we analyzed were correctly identified, and the etiological agent was identified even with the use of the single sequencing direction. In almost all cases, the species identified was predictable, but in some cases we identified unusual bacteria such as *D. tsuruhatensis* and *A. urinaeequi*.

For health management strategy, 16S rDNA has been useful in subtyping virulent bacterial strains associated with outbreaks and can provide some additional information on the prevalence of endemic strains (Sacchi et al., 2002a, b). Moreover, 16S rDNA sequencing has been crucial in the identification of novel and rare bacteria associated with infectious diseases (Clarridge, 2004; Woo et al., 2008).

However, this molecular method has some limitations. There are 'blind spots' within some major genera, in which 16S rDNA sequences are not sufficiently discriminative for the identification of certain species. In these circumstances, alternative targets have to be investigated. For example, *groEL* is a commonly used essential gene other than 16S rDNA which is useful for classification and identification of many groups of bacteria, e.g. staphylococci species (Viale et al., 1994)

Moreover, there is no universal agreement about the percentage similarity required to assign a sequence to a particular species or genus. Globally, the similarity levels, that have been proposed, range from 97% for the genus level to 99% for the species level (Drancourt et al., 2000; Janda and Abbott, 2002; Stackebrandt et al., 2002; Bosshard et al., 2003; Harris and Hartley, 2003; Clarridge, 2004). Assignment to a species can be difficult and relatedness is often more easily shown by alignment and drawing a phylogenic tree (Clarridge, 2004)

Finally, the interpretation of sequences depends on the database, which constitutes a critical factor to bear in mind when considering the possibility of error. This may be linked to the large number of sequences deposited and to the errors of databases by misidentified strains (Kawamura et al., 1995; Facklam, 2002; Harris and Hartley, 2003).

Conclusion

Our results demonstrate clearly the interest and feasibility to introduce the 16S rDNA gene sequencing method in the clinical specimen protocol in Morocco. Moreover, combination of conventional techniques and molecular approach will improve bacteriological diagnosis and allow specific and efficient identification of pathogenic bacteria, limit nosocomial infections and save human lives.

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Full Length Research Paper

Biogenic silver nanoparticles by *Aspergillus terreus* as a powerful nanoweapon against *Aspergillus fumigatus*

Neveen M. Khalil

Botany Department, Faculty of Science, Cairo University, Giza 12613, Egypt.

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In the past few decades, nanoparticles have emerged as a field in biomedical research. Four isolated *Aspergillus* species were tested for extracellular synthesis of silver nanoparticles using their cell free filtrate (CFF). Silver nanoparticles of the most potent producer, *Aspergillus terreus*, were further characterized. Transmission electron microscope (TEM) and atomic force microscope (AFM) revealed their spherical shape, homog eneity and size range between 20 and 140 nm. X-ray diffraction (XRD) showed the crystalline nature of the biogenic silver nanoparticles. Fourier transform infra-red (FTIR) spectroscopic analysis indicated that the coordination behaviors between amino groups of the secreted fungal proteins and other functional groups present in the CFF may be liable for the reduction of silver ions to form stabilized protein-capped silver nanoparticles. They were stable in aqueous solution for four months of storage at room temperature under dark conditions. The biogenic silver nanoparticles showed remarkable antifungal activity against the human pathogenic fungus *A. fumigatus*. The spore cell wall, plasma membrane and the inner constituents were damaged as shown by TEM. Furthermore, comet assay proved high breakage of DNA.

Key words: Silver nanoparticles, biosynthesis, fungi, antifungal, comet assay.

INTRODUCTION

Nanotechnology provides a good platform to modify and develop the important properties of metal in the form of nanoparticles having promising applications in diagnostics, biomarkers, cell labeling, contrast agents for biological imaging, antimicrobial agents, drug delivery systems and nanodrugs for treatment of various diseases (Marcato and Duran, 2008; Singh and Singh, 2011). The nanosize of material results in specific physicochemical characteristics different from those of the bulk materials or larger particles. This effect is mainly credited to high surface-area-to-volume ratio, which results in increased reactivity; hence, the nanoscale materials are more advantageous than their bulk materials. The metallic nano-particles such as copper, titanium, magnesium, zinc, gold and alginate have a strong bactericidal potential owing to their large surface-area-to-volume ratio (Gu et al., 2003; Ahmad et al., 2005). Among all, silver nanoparticles have proved to be the most effective antimicrobial agent against bacteria, viruses and other eukaryotic microorganisms (Gong et al., 2007).

In fact, the use of bulk silver as an antimicrobial dates back to ancient times when water and wine were stored in silver vessels to prevent spoilage. In the 17th century, silver nitrate was used to heal ulcers and chronic wounds. It was further used in the 19th century in the treatment of burns and prevention of ophthalmic diseases in newborns. In the beginning of the 20th century, Barns recognized that silver nitrate was caustic for the eyes of newborns and he invented agyrol, a protein-stabilized silver colloid. Around that same time, a similar colloidal

E-mail: neveen@sci.cu.edu.eg or neveenmmmkhalil@yahoo.com. Tel: +201003643976. Fax: +20237340077.

silver solution was commercialized as collargol. These colloidal silver preparations were early nanosilver formulations (Silver et al., 2006; Nowack et al., 2011).

When silver nanoparticles are chemically produced, three main components are needed: a silver salt (usually $AgNO_3$), a reducing agent (e.g., sodium borohydride) and a stabilizeror capping agent (e.g., polyvinylpyrrolidone) to control the growth of the nanoparticles and prevent them from aggregating (Ledwith et al., 2007). In the case of the biological synthesis of silver nanoparticles, the reducing agent and the stabilizer are replaced by molecules produced by living organisms. These reducing and/or stabilizing compounds can be present in bacteria, fungi, yeasts, algae, or plants. The reduction can happen enzymatically or non-enzymatically and the stabilizer or capping agent is in many cases a protein (Gade et al., 2010; Narayanan and Sakthivel, 2010). One of the main advantages of biogenic silver compared to chemically produced nanosilver is its green synthesis avoiding organic sol-vents and toxic reagents. Moreover, the biological produc-tion of silver nanoparticles converts a waste stream into a product with an added value as biocide, catalyst or biosensor (Hennebel et al., 2009).

When compared with bacteria, fungi have been known to secrete much higher amounts of bioactive substances, which made fungi more suitable for large scale production (Narayanan and Sakthivel, 2010). In addition, the extracellular biosynthesis using fungi could also make downstream processing much easier than bacteria (Mohanpuria et al., 2008).

The present work was aimed at green production of silver nanoparticles by some species belonging to genus *Aspergillus*, due to its prevalence in the natural environment, ease of cultivation on laboratory media and economic importance. The aim was also extended to characterize the biosynthesized silver nanoparticles by the most potent producer *Aspergillus terreus*. In addition, the antimicrobial activity of these nanoparticles against the human pathogenic fungus *A. fumigatus* was studied.

MATERIALS AND METHODS

Isolation of fungal strains

Fungal species used in this study were isolated from an agricultural soil in Giza, Egypt. Soil samples were used as inoculums for soil dilution plate method. Fungal colonies were purified and identified by morphological and microscopic examinations (such as color, texture of mycelia, spore formation pattern, etc.) as described by Raper and Fennel (1965). Fungal species were grown on Potato Dextrose Agar (PDA) slants at 28°C for 4 days. Spores of each strain were harvested and stored at 4°C in sterilized spore suspension buffer containing 0.9% (w/v) NaCl and 1% (v/v) Tween-80.

Synthesis and detection of silver nanoparticles

The stock spore suspension of each fungal species (*Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus niger* or *Aspergillus terreus*) was inoculated in 100 ml of malt extract glucose yeast extract peptone (MGYP) medium (0.3% malt extract, 1.0% glucose, 0.3% yeast extract, 0.5% peptone; pH 6.8) in 250 ml Erlenmeyer flasks. The culture was incubated for 24 h at 28°C in a shaking incubator (150 rpm). This starter culture was used as an inoculum (10 %) for the same medium at the same culture conditions except for three days of incubation. The fungal mycelium was separated from culture medium by centrifugation (5000 rpm, 15 min and 4°C) and washed thrice with sterilized deionized water. The biomass was then autolysed to release the intracellular enzymes and other components into the aqueous solution. This was carried out by suspending 10 g fresh biomass in 100 ml sterilized deionized water and incubated at 28°C for 72 h in a shaking incubator (150 rpm). The contents of the flask were filtered through Whatman filter paper no. 1 and the cell free filtrate (CFF) was obtained. For the biosynthesis of silver nanoparticles, 50 ml of CFF were mixed with 10 ml of 10 mM $AgNO_3$ and incubated at 28°C in a shaking incubator at 150 rpm in the dark.

The reduction of silver ions to silver nanoparticles (by *A. flavus, A. nidulans, Aspergillus niger* or *A. terreus*) was noticed by UV-visible spectrophotometer (Perkin-Elmer, Hitachi 200) at 420 nm. *A. terreus* had highest absorbance rate. Silver nanoparticles produced by this species were chosen for further experiments.

Characterization of silver nanoparticles

FTIR spectrum and X-ray diffraction of freeze dried silver nanoparticles powder were studied using a Fourier Transform Infrared spectrometer (FTIR 6100) and an X-ray diffractometer (Type PANalyticalX'Pert PRO diffractometer) with *CuKα* radiation (λ = 0.15408 nm), operated at 30 mA and 45 kV. Morphology of the synthesized silver nanoparticles was observed by a Jeol JEM-1400 transmission electron microscope (TEM). Further morphological analyses of the particles were conducted by atomic force microscopy (AFM) imaging using a Shimadzu Wet-SPM (Scanning Probe Microscope) in a non-contact mode.

Antifungal activity

The ability of silver nanoparticles to inhibit spore germination of *A. fumigatus* was tested. The minimum inhibitory concentration (MIC) test was carried out. The concentrations of spore suspensions were determined in a hemocytometer and adjusted to 2×10^6 spores/ml. Fifty microlitres of spore suspension were transferred to each well of a microtiter plate containing 100 µl liquid Czapek-Dox medium with different concentrations of silver nanoparticles. Deionized water and amphotericin B replaced silver nanoparticles and were used as negative and positive controls, respectively. The plate was incubated at 30°C for 16 h. All tests were conducted in replicates. Spores were considered to be germinated when the germ tube extended to at least twice the length of the spore itself (Griffin, 1994). Germinated spores were counted using a hemocytometer. About 100 spores per replicate were observed to detect spore germination. MIC was determined as the lowest concentration of silver nanoparticles that inhibits spore germination.

The effect of the tested silver nanoparticles on the ultrastructure of *A. fumigates* spores was studied. The control fungal spores at zero time and after 36 h and those treated with silver nanoparticles for 36 h were isolated, fixed and TEM was performed using a Jeol JEM-1400 transmission electron microscope.

Alkaline single-cell gel electrophoresis (SCGE) or alkaline comet assay was performed to elucidate the action of silver nanoparticles on DNA of fungal spores. The method followed was adopted from Miloshev et al. (2002) and Nemavarkar et al. (2004) with some modifications. Agarose (0.5%) was evenly spread on clean dry frosted slides, and the slides were air-dried. *A. fumigatus* spores or those treated with silver nanoparticles were washed once in sterilized

deionized water and resuspended in a suspending buffer (1 M sorbitol, 25 mM KH_2PO_4, pH 6.5). Approximately, 5 x 10^6 spores were mixed with 0.7% low melting agarose containing 2 mg/ml diluted Macerozyme R-10 (3 ml/100 ml buffer), and the cells were spread evenly on an agarose-coated slide. They were covered with cover slips and incubated for 30 min at 30°C to digest the spore cell wall and to obtain the spheroplast. All further procedures were carried out in a cold room at 10°C to minimize the activity of endogenous enzymes. After removing the cover slips, the slides were incubated in 30 mM EDTA (pH 12.4) to unwind the DNA. The gel-slides were then subjected to electrophoresis in the same buffer at 0.7 V/cm, 30 mA. After electrophoresis, the gels were neutralized by submerging the slides in 10 mM Tris HCl (pH 7.4) for 2-3 min followed by consecutive incubation in 76 and 96% ethanol. The slides were then air-dried and stained in ethidium bromide (2 mg/ml).

A fluorescence microscope (Carl Zeiss) at 400x, with an exciting filter of 515-560 nm and a barrier filter of 590 nm, was used to obtain images and quantify DNA strand breaks in the tested samples. The data are given as tail length, percentage of DNA in tail and tail moment. Tail moment, a commonly accepted unit of DNA damage, is the product of fraction of the DNA in the tail of comet and tail length. Standard errors were calculated on the basis of 30 comets/treatment.

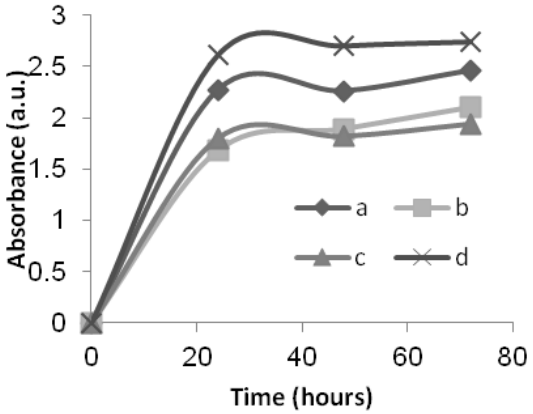

Figure 1. Increase in absorbance at 420 nm for silver nitrate solutions mixed with filtrates from autolysed (a) *A. flavus*, (b) *A. nidulans*, (c) *A. niger* and (d) *A. terreus*.

RESULTS AND DISCUSSION

Isolation of fungal strains

In this study, five species belonging to the genus *Aspergillus* were isolated and purified. They were identified as *A. flavus, A. nidulans, A. niger, A. terreus* and *A. fumigatus*. The former four species were utilized as cell factories for the production of silver nanoparticles. *A. fumigatus* was used as a test organism to study the antifungal properties of biogenic silver nanoparticles.

Synthesis and characterization of silver nanoparticles

Using the supernatant formed from the autolysed biomass allowed the formation of nanoparticles in the absence of any mycelial materials. This resulted in a clearer solution and complications due to nanoparticle/cell interactions were avoided. The extracellular synthesis using CFF is thus more advantageous in isolation of silver nanoparticles from the solution and less number of steps is involved in both synthesis and purification of silver nanoparticles (Kalpana and Lee, 2013).

In this work, color of CFF changed to brown after mixing with $AgNO_3$ for each of the four tested fungal species. The produced brown color indicates the formation of silver nanoparticles. UV-visible spectroscopy at 420 nm (Jain et al., 2011; Alani et al., 2012) was performed to confirm the synthesis of silver nanoparticles in the reaction mixture. As shown in Figure 1, the absorbance increased with incubation time. Development of the brown color was a result of excitation of surface plasmon vibration in the metal nanoparticles and is typical of silver nanoparticles (Gurunathan et al., 2009). Most of the silver nanoparticles (Figure 1) were synthesized by 24 h and there was almost no increase in absorbance after 48 h for the four tested *Aspergillus* species. This loss of activity may be related to a loss of nitrate reductase activity or cofactor availability.

The role of NADH-dependent nitrate reductase from micro-organisms in the biosynthesis of silver nanoparticles was previously discussed (Kumar et al., 2007; Bai et al., 2011). In this study, *A. terreus* was the most potent producer of silver nanoparticles, accordingly, its silver nanoparticles were chosen for further experiments. Stability of silver nanoparticles, produced by *A. terreus*, was detected after four months using UV-visible spectro-scopy at 420 nm. It was found that the nanoparticle solution was highly stable at room temperature under dark conditions, without flocculation. Biological molecules, mostly proteins, stabi-lize biogenic silver nanoparticles which allow functiona-lization of the particles with other biomolecules (Hennebel et al., 2009). Functionalization can increase the antimicro-bial activity by improving the interactions with microor-ganisms (Botes and Cloete, 2010). Chemically produced nanopar-ticles on the other hand, are known to aggregate to larger clusters (Mafune et al., 2000). This decreases their high specific area and therefore also their catalytic and antimicrobial activity.

FTIR analysis of the freeze-dried sample was carried out to identify the possible interactions between silver and bioactive molecules, which may be responsible for synthesis and stabilization (capping material) of silver nanoparticles. The amide linkages between amino acid residues in proteins give rise to well-known signatures in the infrared region of the electromagnetic spectrum (Jain et al., 2011). The FTIR spectrum (Figure 2) revealed a peak at 3423 cm^{-1} (no. 1) which could be attributed to strong stretching vibrations of hydroxyl functional group

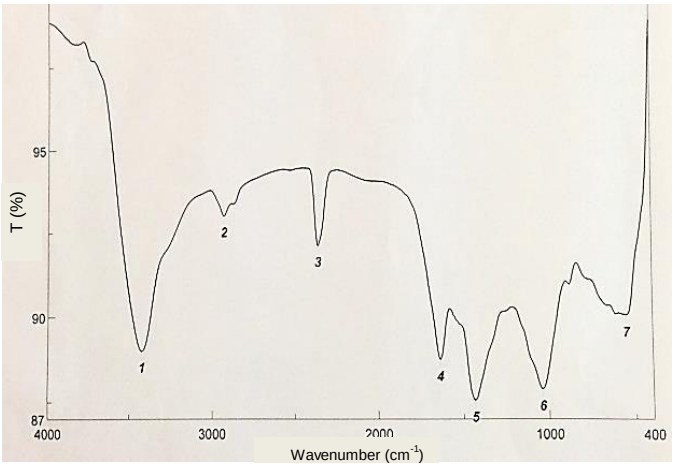

Figure 2. FTIR spectrum of silver nanoparticles synthesized by *A. terreus*.

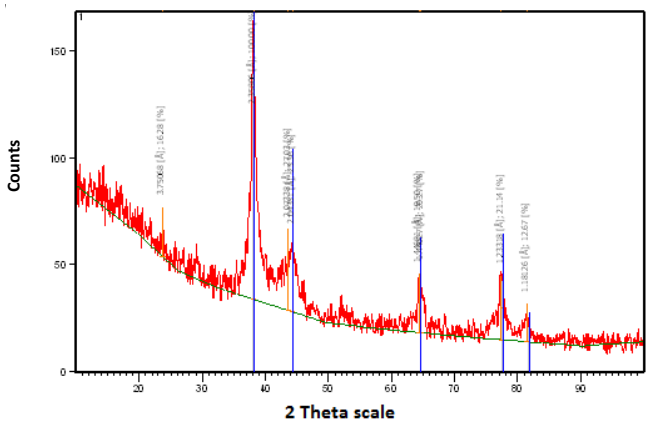

Figure 3. X-ray diffraction (stick pattern) of silver nanoparticles synthesized by *A. terreus*.

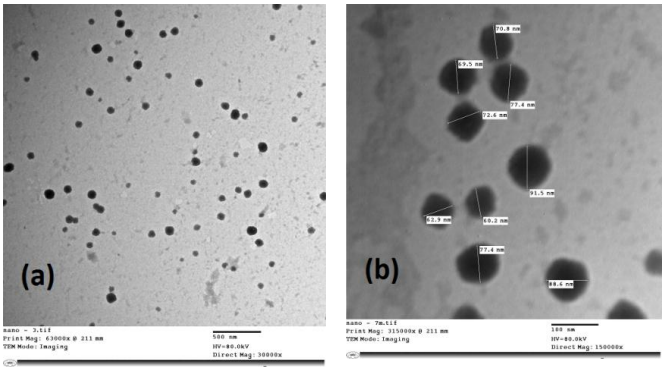

Figure 4. TEM photographs of silver nanoparticles synthesized by *A. terreus*. (a) Low magnification image (scale bar: 500 nm) and (b) high magnification image (scale bar: 100 nm).

(Priyadarshini et al., 2013). The peak no. 5 at 1432 cm^{-1} may be related to COO- symmetrical stretch from carboxyl groups of the amino acid residues (Gajbhiye et al., 2009). Hydroxyl groups of Tyr residues and carboxyl groups of Asp and Glu residues are the most active functional groups for Ag^{+} reduction and silver nanoparticles anisotropic growth (Xie et al., 2007). The bands no. 6 and 4 at 1039 and 1635 cm^{-1}, respectively, may correspond to -N-H and carbonyl (C-O-) stretching vibrations in amide linkages (amide I and amide II) of proteins (Suresh et al., 2011). The peak no. 2 at 2931 cm^{-1} could be due to C-H stretch of methylene groups of proteins (Ghaseminezhad et al., 2012).

The X-ray diffraction study confirmed the crystalline nature of synthesized silver nanoparticles. Figure 3 shows four distinct peaks at 38.29, 44.51, 64.77 and 77.81° which were assigned to the (111), (200), (220) and (311) lattice planes of face-centered-cubic (fcc) silver, respectively (Gopinath et al., 2013). The obtained diffraction spectrum suggested the presence of silver nanoparticles which is in accordance with the UV-visible spectrum analysis.

TEM analysis of silver nanoparticles (Figure 4) revealed their homogeneity, well dispersion and spherical morphology indicating their stabilization. On the other hand, atomic force microscope imaging (Figure 5) showed silver nanoparticles to be uneven due to the presence of some of the aggregates along with individual nanoparticles. It proved the morphological homogeneity with the grain size falling in the submicron range. Spherical shape of the synthesized silver nanoparticles was confirmed and their size lied mostly in the range of 20-140 nm. A variety of different-sized silver nanoparticles has been reported depending on the biological system used. Also, diverse shapes have been noticed with spherical particles being predominant. Alani et al. (2012) found that silver nanoparticles from *A. fumigatus* had a diameter ranging from 15 to 45 nm meanwhile those from *Streptomyces* sp. showed a narrower size distribution of 15-25 nm. The silver nanoparticles synthesized by *Puccinia graminis* (Kirthi et al., 2012) were shown to have a size between 30 and 120 nm while those from the white rot fungus *Pycnoporus sanguineus* had an average diameter of 52.8-103.3 nm (Chan and Don, 2013).

Antifungal activity

A. fumigatus is a fungal saprophyte that is ubiquitous throughout the world. It is also an opportunistic pathogen of immunocompromised hosts causing invasive aspergillosis, a usually fatal infection. Germination of inhaled conidia (spores) is an early and crucial event in the infection process of *A. fumigates* (Rohde et al., 2002). The antifungal test was performed against this pathogen with different concentrations of silver nanoparticles (from

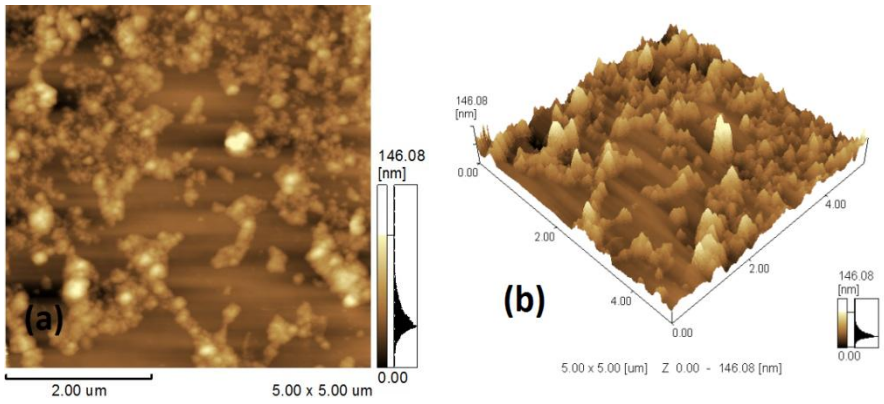

Figure 5. Atomic force microscope images of silver nanoparticles synthesized by *A. terreus* depicting the topography. (a) Top view and (b) 3-D view.

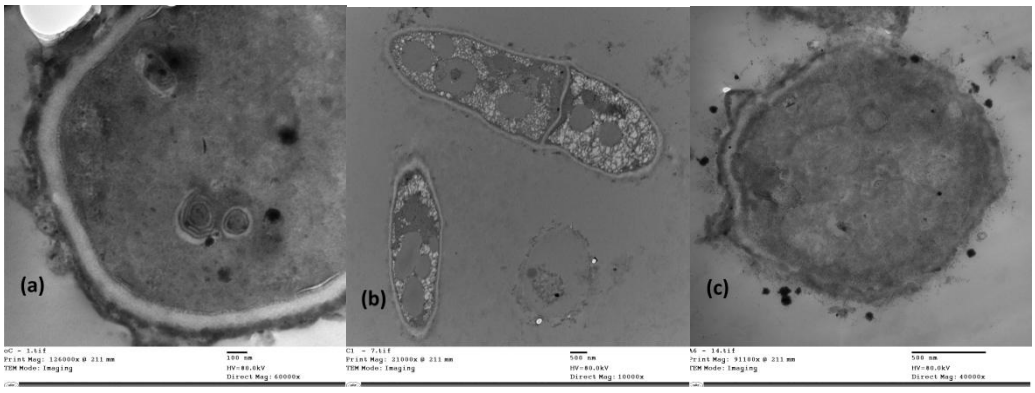

Figure 6. TEM micrographs of cross sections of *A. fumigatus* spores. (a) resting spores, (b) control spores after 36 hours of incubation in Dox's medium and (c) spores after 36 hours of incubation in Dox's medium containing silver nanoparticles (15 µg/ml).

1.88 to 94 µg/ml). The results revealed that the MIC of silver nanoparticles against germination of spores of *A. fumigatus* may be estimated to be between 7.52 and 11.28 µg/ml. The antifungal drug amphotericin B was used as positive control and it showed an MIC between 70 and 75 µg/ml. The significant antifungal activity shown by the biogenic silver nanoparticles suggests their potential against infections caused by *A. fumigatus*. Several studies proved the antimicrobial activity of silver nanoparticles (Kim et al., 2007; Jain et al., 2010; Lamsal et al., 2011; Kalpana and Lee, 2013; Chan and Don, 2013). Kora and Arunachalam (2012) compared the antibacterial effect of silver nanoparticles against some bacteria with erythromycin, a commonly used antibiotic. The antibacterial activity of silver nanoparticles was higher in most cases. Meanwhile, Li et al. (2012) studied the antimicrobial activity of silver nanoparticles against some bacteria, yeasts and fungi. They used silver nitrate as a control. In all tested microorganisms, silver nanoparticles showed higher inhibitory effect than silver nitrate.

It was thought advisable to study the effects of the biogenic silver nanoparticles on spores of *A. fumigatus via* TEM analysis. The main abnormalities noted *via* TEM study (Figure 6) was the alterations in the morphology and complete collapse of the spores after 36 h of exposure to the biogenic silver nanoparticles. The typical echinulate surface and inner constituents of the resting spore is shown in Figure 6a. Germinating spores (Figure 6b), after 36 h of incubation in Dox's medium, seemed to shed the outer surface layer before the cells start to swell, germinate and multiply. On the other hand, the spores incubated in Dox's medium containing silver nanoparticles for 36 h (Figure 6c) looked different. Silver nanoparticles appear to accumulate, adhere on and penetrate the outer surface of spore. Parts of the cell wall, plasma membrane and the inner constituents of the spores were obviously damaged which may be due to the toxic effect of the silver nanoparticles.

The alkaline SCGE technique was applied to *A. fumigatus*

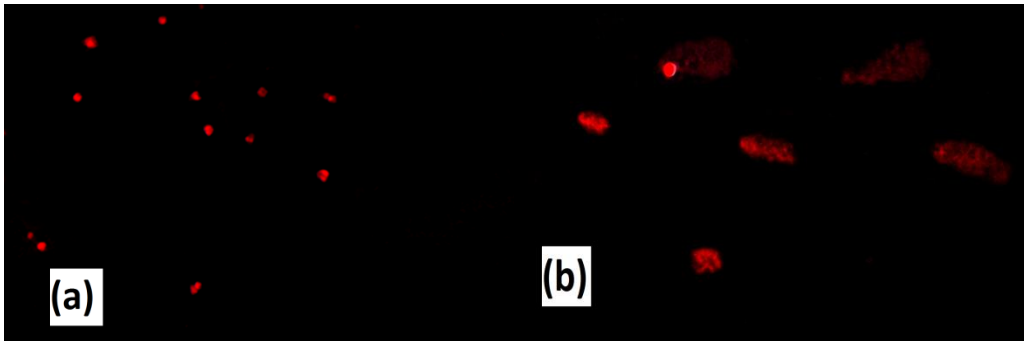

Figure 7. Comets from *A. fumigatus* spore suspension. (a) Control spores and (b) spores treated with silver nanoparticles (15 µg/ml).

Table 1. DNA damage parameters of comet assay of *A. fumigatus* spore cells exposed to silver nanoparticles.

Group	Tail length (px)	DNA in Tail (%)	Tail moment
Control	3.00 ± 0.54	21.54± 2.78	0.61± 0.09
Treated	13.87 ± 1.92	35.59 ± 3.14	5.39 ± 1.12

Data presented are mean ± standard error. 1 px = 0.24 µm.

spores to follow the silver nanoparticles-induced DNA breaks. A clear-cut nearly spherical image was seen in control spore cells illustrating undamaged DNA (Figure 7a). DNA was still contained in the cells which were protected by cell walls. On the other hand, a characteristic picture of comets was obtained for spores treated with silver nanoparticles (Figure 7b), which denotes DNA damage. The results in Table 1 reveal the increase of tail length, percentage of DNA in tail and tail moment of spores subjected to silver nanoparticles. There was a nearly 9-fold increase in tail moment due to presence of silver nanoparticles.

Some reports explained that silver nanoparticles attack bacterial cell by anchoring and penetrating cell wall. As a consequence, a structural change in cell membrane takes place which leads to an increase in cell permeability. Hence, uncontrolled transport through cytoplasmic membrane followed by cell death is the fate (Morones et al., 2005; Sondi and Salopek-Sondi, 2007). Kim et al. (2007) attributed the antibacterial mechanism of silver nanoparticles to the formation of free radical-induced membrane damage. Klueh et al. (2000) proposed that silver nanoparticles inhibit bacterial growth by binding to thiol groups (-SH) in enzymes, which deactivates the enzymes. They also hypothesized that the bactericidal activity of silver nanoparticles is due to silver ions which enter the cell and intercalate between the purine and pyrimidine bases of DNA. These base pairs showed disturbing effect on the hydrogen bonding between the two antiparallel strands, leading to denaturation of DNA. Accordingly, cell division and DNA replication are prevented which ultimately leads to cell death.

In conclusion, four *Aspergillus* species were isolated from soil and used in the production of silver nanoparticles. Using a simple autolysis method on the biomass, the released intracellular materials were used in the cell-free biosynthesis of silver nanoparticles from silver nitrate solution. *A. terreus* produced highest concentrations of silver nanoparticles. These biogenic silver nanoparticles were highly stable for a long storage period. They showed strong antifungal activity against the human pathogenic fungus *A. fumigatus*. Thus, it could be recommended that extracellular synthesis of silver nanoparticles from *A. terreus* could be used in developing novel antifungal agents which may find potential applications in the drug industry.

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Full Length Research Paper

Phytochemical and antibacterial activity of *Securidaca longepedunculata* on selected pathogens

Ndamitso, M. M.[1]*, Mohammed, A.[2], Jimoh, T. O.[1], Idris, S.[1], Oyeleke, S. B.[2], and Etsuyankpa, M. B.[3]

[1]Department of Chemistry, Federal University of Technology, Minna, Niger State, Nigeria.
[2]Department of Microbiology, Federal University of Technology, Minna, Niger State, Nigeria.
[3]Centre for Preliminary and Extramural Studies, Chemistry Section, Federal University of Technology, Minna, Niger State, Nigeria.

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Securidaca longepedunculata **family Polygalaceae is a tropically distributed medicinal plant. Antibacterial activity of chloroform, methanol and aqueous extracts of the roots and leaves of the plant against some selected microorganisms were shown using standard Kirby-Bauer disk diffusion method. The plants extracts showed inhibitory activity against the tested organisms. The diameter of zones of inhibition exhibited by all the extracts was between 15 and 20 mm. The methanol and the chloroform extracts of the leaves compared favorably with ampliclox capsule used as a standard control. The minimum inhibitory concentrations (MICs) of the extracts ranged from 0.591 to 6.25 mg/ml while the minimum bactericidal concentrations (MBCs) ranged from 1.56 to 6.25 mg/ml. Chromatography of methanol and aqueous extracts of the leaves revealed two major spot. The phytochemical screening of the extracts revealed the presence of alkaloids, flavonoids, saponins, tannins, cardiac glycosides, anthraquinones, steroids, balsams and reducing sugars. The study scientifically validates the use of this plant in traditional and ethnomedicine and these extracts may be a potential source of future antibacterial drugs against enteric organisms.**

Key words: Phytochemical, antibacterial activity, *Securidaca longepedunculata*.

INTRODUCTION

Resistance to antibiotics has been so tremendous that, incidences of outbreaks of multi-drug resistant bacteria in the past decades increased dramatically with no effective antibiotics to treat them (Walsh and Ames, 2004). In addition, widespread of diseases such as cancer, tuberculosis, typhoid fever, malaria, influenza, skin rashes and cardiovascular diseases coupled with high poverty level in developing countries like Nigeria has made investment and investigations on herbal plants an attractive endeavour in human healthcare. This is because most of the available synthetic medicines are too expensive for most patients (Eisenberg et al., 2005). In the past decade, there has been renewed attention and interest in the use of traditional medicine globally (WHO, 2002). Traditional medicine was a source of many important medical pharmaceuticals (Gilani and Atta-ur-rahman, 2005). Recently, plant derived compounds offer an additional potential source of new antimicrobial, anticancer and anti-HIV agents (Gurib-Fakim et al., 2005).

Seccuridaca longepedunculata is a shrub of about 10 cm high, 2 to 9cm long and 0.5 to 2.5cm broad leaves commonly found in the entire Sudano – Zambezian zone.

*Correcponding author. E-mail: ndamitso@yahoo.com. Tel: +2348032904468.

The plant belongs to the family Polygalaceae. Its leaves are oblancelate and obtuse at apex. The flowers are purple or blue in colouration and the seeds are winged (Abdullahi et al., 2003). In northern Nigeria, the Nupe and the Hausa tribes utilize *S. longepedunculata* ethnomedicinally as a remedy for numerous human and animal ailments (Deeniand and Sadiq, 2002). According to Dapar et al. (2007), the aqueous extracts of its roots are used as pyschopharmaceautical agents. It is also used as a sexual boost for men (Menecke and Mulhall, 1999; Nair and Chanda, 2006). This plant is also used for the treatment of every conceivable ailment such as headache, rheumatism, tuberculosis, cancer, venereal diseases, diabetes as well as abortifacient (Avhurengwi and Walter, 2006) and probably that is why the Hausas refer to it as “uwar magunguna” (the mother of all medicines). Therefore, the objective of this study was to authenticate the claims of the traditional healers on this plant which will form the basis for further research.

MATERIALS AND METHODS

Collection and preparation of plant materials

The leaves and roots of the plant were collected from the Abattoir, behind Ahmadu Bahago Secondary School in Bosso Local Government Area, Minna, Niger State. Identification was done by Professor Z. I. E. Ezenwa of School of Agriculture and Agricultural Technology, Department of Soil Science, Federal University of Technology, Minna. The samples were washed with distilled water to remove earthy materials, dried at ambient temperature in the laboratory to avoid heat destruction of the active components before powdering. The powdered samples were then put in clean dried cellophane bags and kept in a cool dry place for further use.

Plant extraction

100 g of each powdered material was weighed and extracted with 300 cm^3 of chloroform by refluxing for six hours and filtered. The marc was extracted with 300 cm^3 of methanol in the same way as that of the chloroform. After drying, the marc was extracted with distilled water using reflux. The various extracts obtained were evaporated to dryness on a steam bath. The dried extracts were weighed, kept in well labeled sterile specimen bottles and stored in a refrigerator at 4°C until required.

Phytochemical screening

The crude extracts of the samples were subjected to phytochemical tests to determine their chemical constituents using standard methods described by Evans and Trease (1989) and Sofowora (1982). The tannins were determined by suspending 3 g of each extract in 6 cm^3 of distilled water after which it was filtered and iron (III) chloride reagent was added. For the cardiac glycosides, Keller-Killiani’s test (Trease and Evans, 1989) was adopted by taking 0.5 g of the extract and adding 2 cm^3 of acetic acid plus H_2SO_4. The alkaloids were tested for by taking 0.5 g of the aqueous extract in 5 cm^3 1% HCl. This was boiled, filtered and Mayer's reagent added (Trease and Evans, 1989) while for saponins, the extracts were subjected to frothing test. Haemolysis test was performed on the frothed extracts in water to remove false positive results (Sofowora, 1993). Anthraquinones were tested for by treating 5.0 g of each extract with 10 cm^3 of benzene, filtered and ammonia solution was added (Sofowora, 1993). The presence of flavonoids were determined using Shinoda’s test for flavonoids by dissolving 0.5 g of each extract in 5 cm^3 of ethanol, warmed and filtered. This was followed by the addition of magnesium chips to the filtrate and few drops of concentrated HCl (Trease and Evans, 1989). The steroidal constituents were determined when 2.0 g of each extract was treated with 2 cm^3 of acetic acid, warmed and cooled in ice followed by careful addition of concentrated H_2SO_4 (Sofowora, 1993). The presence of reducing sugar was established by Fehling’s test for reducing sugar. For each extract, 0.5 g was dissolved in distilled water and filtered. The filtrate was heated with 5 cm^3 of equal volumes of Fehling’s solutions A and B (Sofowora, 1993).

Test bacteria

The test bacteria used in this study were isolates of *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhii* obtained from their stock cultures in Microbiology Department Laboratory, Federal University of Technology, Minna. The bacterial isolates were cultured on nutrient agar and incubated at 37°C for 24 h. These were repeatedly sub-cultured in order to obtain pure isolates while morphological and biochemical tests were carried out to ensure proper identification. In this case, a loopful of test organism was inoculated on nutrient broth and incubated for 24 h. A volume of 0.2 ml from the previously cultured organism was dispensed into sterile nutrient broth and incubated for 3 h to standardize the culture to 1.0×10^6 cfu/ml (Oyeleke et al., 2008).

Antibacterial activity

The standard Kirby-Bauer disk diffusion method described by NCCLS (2002) was adopted for the antibacterial activities of the extracts. Stock solutions of 25 mg/ml were prepared from the dried extracts. Test bacteria were sub-cultured onto brain-heart infusion agar (Becton Dickinson Comp., USA) and incubated at 37°C for 24 h after which 3 colonies were isolated with inoculating loops, transferred to three tubes of sterile saline and vortex thoroughly. The bacterial suspensions were compared and adjusted to 0.5 McFarland standards to prepare culture stocks (about 10^6 cfu/cm^3). Within 15 min, sterile cotton swabs were dipped into the bacterial suspensions and streaked over sterile plates containing nutrient agar and left for a while to set. Sterile filter paper (6 mm in diameter) was impregnated with 15 µl of extract (7.5 mg/disc) from previously prepared stock of 25 mg/ml. After 20 min, the plates were gently turned upside down and incubated at 37°C for 24 h. The diameters of inhibition zones (in mm) were measured and recorded. Commercial antibiotic (ampiclox) was applied as positive control.

Determination of minimum inhibitory concentration (MIC)

MICs of the extracts were recorded as the lowest concentrations of the extracts that inhibited the growth of the microorganisms.

Determination of minimum bactericidal concentration (MBC)

MBCs of the extracts were determined by sub-culturing the test tubes that showed no visible turbidity after the incubation of the batch test tubes in the MICs determination on nutrient agar plates which were then incubated at 37°C for 24 h. The concentration that showed no visible growth after incubation was taken as the MBC (Suffredini et al., 2004; Doughari et al., 2007).

Table 1. Phytochemical constituents of extracts from roots and leaves of *S. longepedunculata.*

Chemical compound	Chloroform extract		Methanol extract		Aqueous extract	
	Roots	Leaves	Roots	Leaves	Roots	Leaves
Saponins	-	-	+	++	++	++
Tannins	-	+	-	+++	-	+++
Flavonoids	+	+	+	+	+	+
Steroids	-	+	+	-	-	-
Cardiac glycosides	-	+++	-	+++	-	+++
Alkaloids	-	-	+	+	+	+
Anthraquinones	-	-	++	++	-	++
Reducing sugars	+	+	+	+	+	+

Table 2. Antibacterial activities of the crude extracts of *S. longepedunculata* (showing zones of inhibition in mm).

Bacteria	Chloroform extract		Methanol extract		Aqueous extract		Control (Ampiclox)
	Roots	Leaves	Roots	Leaves	Roots	Leaves	
Escherichia coli	-	17	-	15	20	15	23
Salmonella typhi	-	16	-	18	-	15	23
Pseudomonas aeruginosa	-	18	-	19	-	-	18

- = Not active.

Thin layer chromatographic determination

The R_F values of the separated components obtained by chromategraphic separation of the crude extracts were determined using mini thin layer plates prepared by using microscope slides while the macro-plates were prepared using 20 × 10 cm glass slides. A mobile phase made up of the mixture of ethyl acetate, methanol and distilled water in the ratio of 12:1.5:1.0 was made, put in a glass tank, closed and allowed to stand for about 10 min. The plates were inserted into the tank with the origin of spots towards the bottom of the tank but above the solvent and covered tightly in order to allow the solvent get close to the top. They were removed, dried in the oven and the distances moved by the solvent and the extracts measured. The separations obtained by the macro-plates were scraped, dissolved in appropriate solvents, evaporated and packaged in airtight containers for further use.

RESULTS AND DISCUSSION

Table 1 shows the phytochemical constituents of the extracts. While flavonoids and reducing sugars were fairly present in all the extracts, tannins, were highly present only in the methanolic and aqueous leaves extracts. Other constituents were present in the methanolic roots and leaves extracts except tannins, steroids and cardiac glycosides.

The presence of alkaloids and flavonoids in both methanol and aqueous extracts (Table 1) revealed the efficacy of the plant against the diseases that they are used locally for. The presence of flavonoids in the extracts also confirms the assertion of Ingrid and Mathias (2006) who said that *S. longepedunculata* is a highly antidiabetic plant. The antidiabetic activity of this plant is probably due to the ability of flavonoids to inhibit α-amylase activity which regulates the amount of glucose in the blood. The presence of saponins and glycosides in reasonable quantities also justified the traditional use of the plant in the treatment of tuberculosis and diabetes (Abdullahi et al., 2003).

Table 2 shows the zones of inhibition of the crude extracts against the test organisms. The chloroform and methanolic roots extracts showed no activity and the aqueous roots extract was only active against *E. coli*. The chloroform, methanolic and aqueous leaves extract showed varied activities against the test organisms.

The strong activity of the roots and leaves extracts suggested that this plant could be used for the treatment of infections caused by the test organisms except *P. aeruginosa* that was resistant to activity of the aqueous leaves extract (Table 2). The root extracts of the three solvents used were inactive against the test organisms except *E. coli* while the control (ampiclox) had the highest inhibitory activity against any of the test organisms.

These results were however, higher than the respective 14.0 and 8.0; 10.0 and 10.0 mm reported by Yahaya et al. (2012) as the zones of growth inhibition of *Salmonella typhi* by the mehanolic (stem extract) and methanolic or aqueous (leaves extracts) of *Combretum glutenosum*. In addition, none of these values was lower than the 7 mm reported for *Andrographilis paniculata* although they were lower than the 23 mm reported for *Euginia jambolana* (Muhamed et al., 2010). However, the recorded zones in this study for *P. aeruginosa* were lower than the respective 18 mm reported for *A. paniculata* and *E. jambolana* by Muhamed et al. (2010) but similar to the 15

Table 3. The minimum inhibitory concentrations (MIC) in mg/ml of the crude extracts of *S. longepedunculata.*

Bacteria	Chloroform extract		Methanol extract		Aqueous extract	
	Roots	Leaves	Roots	Leaves	Roots	Leaves
Escherichia coli	-	1.563	-	1.563	6.25	1.563
Salmonella typhi	-	0.591	-	0.591	-	6.25
Pseudomonas aeruginosa	-	0.591	-	1.563	-	-

- = Not active.

Table 4. The minimum bactericidal concentrations (MBC) of the crude extracts of *S. longepedunculata* in (mg/ml).

Bacteria	Chloroform extract		Methanol extract		Aqueous extract	
	Roots	Leaves	Roots	Leaves	Roots	Leaves
Escherichia coli	-	15.63	-	15.63	62.5	15.63
Salmonella typhi	-	5.91	-	5.91	-	62.5
Pseudomonas aeruginosa	-	5.91	-	15.63	-	-

- = Not active.

mm reported for the acetone extract of *Aloe vera* by Arun and Muthuselvam (2009). These antibacterial effects of *S. longepedunculata* were attributable to the presence of the active phytochemicals like tannins, saponins, alkaloids and glycosides in the extracts (Enwerem et al., 2001, 2003).

Dapar et al. (2007) reported that tannins form complexes with proteins through hydrogen and covalent bonds as well as other hydrophobic effects which inhibit cell protein synthesis. The presence of these phytochemicals therefore has been adduced for the significant antibacterial activity of the extracts. Also, the protein-precipitating and vasoconstriction effects of tannins help in preventing ulcer development (Dahiru et al., 2006) and this is one of the ailments that this plant is used to treat locally in the study area.

The diuretic and antibacterial activity of plant extracts containing flavonoids have been documented (Enwerem et al., 2001, 2003). The activities of the methanolic extracts and chloroform fractions of the leaves were not significantly different and this showed that both extracts can be exploited for antibacterial actions.

Tables 3 and 4, respectively, show the minimum inhibitory and minimum bactericidal concentrations of the extracts. The test bacteria were inhibited at concentrations ranging from 0.591 to 6.25 mg/ml while the minimum bactericidal conetrations ranged from 5.91 to 62.50 mg/ml. Just in line with the observations of Karaman et al. (2003), the aqueous root extracts had the highest MIC values but these were all lower than the 256 mg/ml reported as the minimum inhibitory concentration for the ethanolic extract of *Iresine herbstii* by Bussmann et al. (2010) but were higher than the respective 16, 32 and 32 µg/ml reported as the MIC values for *E. coli*, *P. aeruginosa* and *S. typhi* by Hassan et al. (2009) for *Poygonum hydropiper*. The lowest MBC of 5.91 mg/ml recorded for *P. aeruginosa* in this work was higher than the 256 µg/ml reported as the MIC of *Dioscorea bulbifera* extracts against this organism (Victor et al., 2012).

Table 5 showed the zones of inhibition of the chromategraphic fractions of the extracts against the test organisms. The chloroform and methanolic roots fractions showed no activity while the aqueous roots fractions were only active against *E. coli*. The leaves extracts of the three solvents showed various degrees of activity against the test organisms.

The TLC fractions obtained from the leaves had R_F value of 0.66 for the aqueous extract while that obtained from the methanol extract of the leaves was 0.78. The roots and leaves chloroform extracts as well as the roots methanol and aqueous extracts had no noticeable spots in the solvent mixture but chlorophylls were observed at the solvent front. The scrapped fractions exhibited antibacterial activities against the test organisms and one of the fractions from the aqueous leaves extracts had the highest effect against *S. typhi*. The fractions however, showed lower antibacterial activities against the test organisms (Table 5) than the crude extracts (Table 2). The lower antibacterial activities of the fractions than the crude ones might have been due to the synergic effects of the active components in the crude extracts thus agreeing with the reports of Harborne (1984) and Oyeleke et al. (2008) who said that activities of plant extracts could change after fractionation making the obtained pure component to lack the activity of the original crude extract.

Conclusion

It could be inferred that these plant extracts could be useful in the industrial manufacture of drugs used in the

Table 5. Antibacterial activities of the crude chromatographic fractions of *S. longepedunculata* showing zones of inhibition in (mm).

Bacteria	Chloroform extract		Methanolic extract		Aqueous extract		Control (Ampiclox)
	Roots	Leaves	Roots	Leaves	Roots	Leaves	
Escherichia coli	-	13	-	15	16	17	23
Salmonella typhi	-	17	-	17	-	16	23
Pseudomonas aeruginosa	-	12	-	16	-	-	18

- Not active

chemotherapy of some microbial infections. Thus, the present study provides some information on the phyto-chemical and antibacterial investigation of *S. longepedunculata* which paves way for further research to identify the active compounds responsible for the biological activity of the plant.

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Full Length Research Paper

Informant consensus factor and antimicrobial activity of ethno medicines used by the tribes of wayanad district kerala

A. G. Devi Prasad[1]*, T. B. Shyma[1] and M. P. Raghavendra[2]

[1]Department of Studies in Environmental Science, University of Mysore, Manasagangotri, Mysore-570 006. Karnataka, India.
[2]Department of Microbiology, Maharani's College, Mysore, India.

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The ethno botanical investigation of medicinal plants used by the Kurichia, Kuruma, Kattunaika, Adiya and Paniya tribes of Wayanad district, Kerala were recorded. One thousand (1000) ethno medicines derived from 500 plants used by the tribal medical practitioners were documented. Of this, 10 species were frequently used for treating various infectious diseases. An informant consensus factor was calculated for 10 species which are being frequently used. The leaf extracts of these plants were screened for antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Aspergillus niger.* Among the plants screened *Garcinia gummi gutta* (Clusiaceae) was found to possess highly significant antibacterial activity and significant antifungal activity was observed in *Nothapodytes nimmoniana* (*Icacinaceae*) which possess highest antifungal activity. These two species have shown the highest informant consensus factor values.

Key words: Medicinal plants, informant consensus factor, antibacterial activity, antifungal activity, ethno botany.

INTRODUCTION

Antimicrobials provide the main basis for the therapy of microbial (bacterial and fungal) infections. However, over use of antibiotics has become the major factor for the emergence and dissemination of multidrug resistant strains of several groups of micro organisms. The worldwide emergence of multi drug resistant *Escherichia coli* and many other β- lactamase producers has become a major therapeutic problem (Khan et al., 2004). For this reason, researchers are turning their attentions to herbal products, looking for new leads to develop better drugs against multidrug resistant microbe strains (Kafaru, 1994).

There are over 275,000 species of flowering plants in the world today (Anonymous, 2000). Various plant parts have been used by man for the treatment of diseases, particularly those caused by micro-organisms. Although hundreds of plants have been tested for antimicrobial properties, the vast majority have not been adequately evaluated especially the tribal medicines. There is a need to screen plants used by the tribal people and document them through scientific validation. Wayanad has a great wealth of medicinal plants and tribal people. The tribes have an ancestral tradition about the use of medicinal plants and there are reviews on different types of medicinal plants used by the Wayanad tribes (Nisha and Sivadasan, 2007; Mini and Sivadasan, 2007; Raji and Raveendran, 2011). The modern civilization has put them on the verge of extinction. To rescue this knowledge, it is necessary to explore the scientific information about these medicinal plants in Wayanad district. In this regard,

*Corresponding author. E-mail: agdprasad@yahoo.com. Tel: 09448033391.

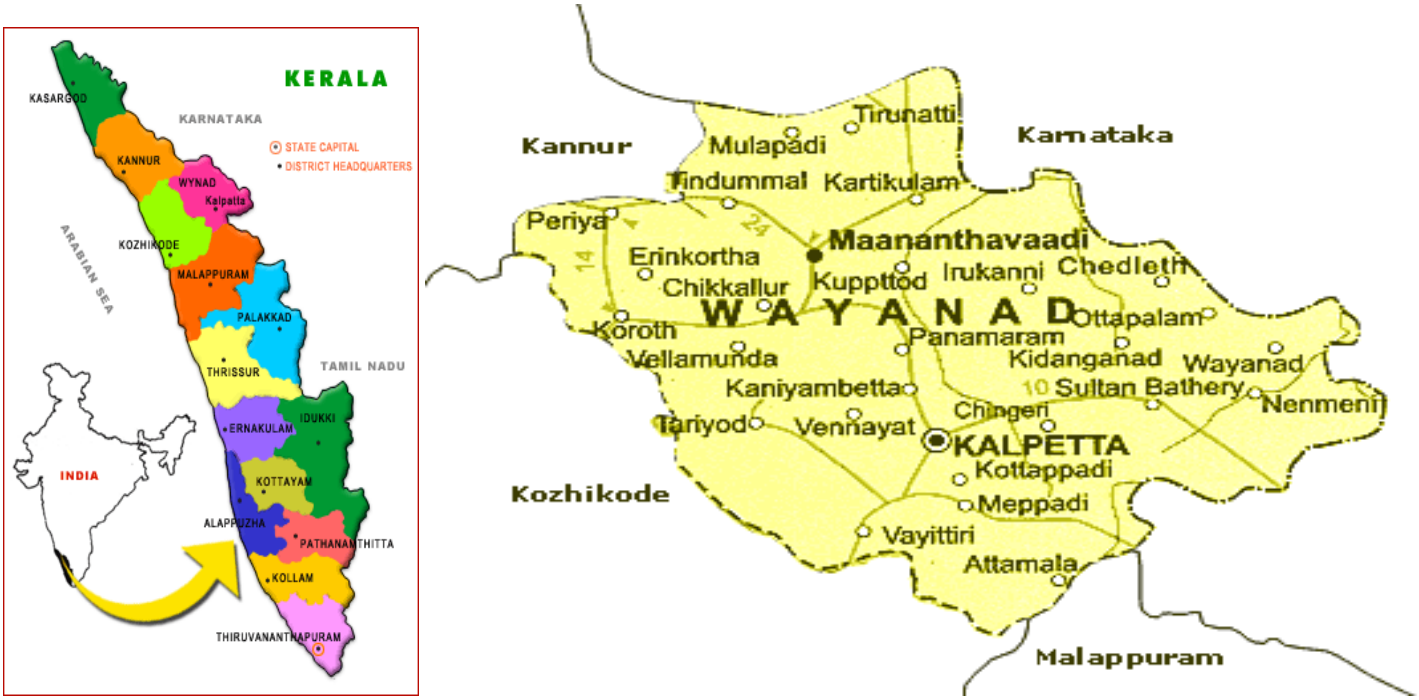

Figure 1. Wayanad district map.

there is ethno directed method of exploitation of these plants. This technique helps in the collection of plants based on the knowledge. According to Cox and Balick (1994) and Cordell (2000), this method plays a fundamental role in biodiversity prospecting. Since time does not allow us to evaluate all existing medicinal plants scientifically selection of the most important taxa is a prerequisite to begin ethno pharmacological, phytochemical and toxicological studies. For this purpose it is necessary to determine the species that are most used to treat a particular illness. A useful tool to find these species is the informant consensus factor (Frei et al., 1998; Heinrich et al., 1998a).

In view of this, the present investigation was undertaken to document the medicinal plants used by the tribes of Wayanad to treat infectious diseases, and also to evaluate the antimicrobial activity considering informant consensus factor.

Study area

The district of Wayanad is situated in the eastern part of Kerala and lies between 11° 27’ and 11° 58’35"N and 75° 47’50" and 76° 26’ 35"E (Figure 1).

Ethnic groups

The district of Wayanad has the largest population of the tribes in Kerala. Wayanad district accounts for one third of the total tribal population in Kerala, which is equivalent to 17.43% of the district population. The total tribal population in Wayanad as per Census 2001 is 136,062. The main tribal groups in Wayanad include Paniya 45%, Kurichiya 17%, Mullu Kuruma 17%, Kattunaikya 10% and other splinter tribal groups 1%.

The name 'paniyan' means 'worker' as they were supposed to have been the workers of non-tribes. They are dark skinned, short stature, long headed with long wavy curly hair and broad nose. The dialect of the paniyan people is paniya language. People of paniya still depend upon the local flora for healing their illness (Raji and Raveendran, 2011). Adiya is one of the slave sects in Kerala. The adiyas speak a dialect of Kannada. Their traditional occupation is agricultural labour. There are mainly distributed in Mananthavady and Vythiri taluks of this district. As their name denote, the kattunayakan were the kings of the jungle regions engaged in the collection and gathering of forest produces. They have all the physical features of a hill tribe. They speak kattunaikyan dialect, which is a mixture of all Dravidian languages, drawing more from Kannada. The kurumas are also known as mullu kuruma. They are mostly found in the Wayanad region. There are three sub-divisions in the kuruma tribal sect: uralIkuruma, 'Mullu Kuruma' and 'Jenu Kuruma' (honey gathering kurumas) tribes. Amongst the kuruma tribes in Wayanad, the Mullu Kurumas consider themselves superior to the other Kuruma sects. The

Kurichias are an agricultural tribal community. They speak Malayalam and use Malayalam script. The Kurichians are rich in their oral tradition.

MATERIALS AND METHODS

Plant collection and ethno botanical interviews

The ethno botanical survey was conducted in the tribal localities of three taluks Mananthavady, Bathery and Vythiri of Wayanad district, Kerala. Field explorations were undertaken during 2006-2010 among the tribal colonies of Mananthavdy, Bathery and Vythiri taluk to identify ethnic groups and the plants used by them as medicines. The confirmation of identification has made in consultation with taxonomic experts of MS Swaminathan Research Foundation, Wayanad and by referring to literature.

A questionnaire was prepared to collect information related to informant consensus factor, method of application, therapeutic use, parts of plants used, name of diseases, symptoms of diseases and causes of diseases, etc.

Informant consensus factor

To estimate use variability of medicinal plants and to determine which plants are particularly interesting in the search for bioactive compounds, the informant consensus factor (F_{ic}) (Heinrich et al., 1998 a) was calculated. This factor estimates the relationship between the number of use reports in each category (n_{ur}) minus the number of taxa used (n_t) and the number of use reports in each category minus 1. F_{ic} is thus calculated using the following formula:

$$F_{ic} = \frac{n_{ur} - n_t}{n_{ur} - 1}$$

The product of this factor ranges from 0-1. A high value close to one indicates the relatively few species used by large proportion of people. While a low value indicates that the informants disagree on the taxa to be used in the treatment within a category of illness (Canales et al., 2005).

Bioassays

The bacterial samples of *Esherichia coli* isolated from stool samples of diseased persons, *Staphylococcus aureus* isolated from infected urine samples and fungal samples of *Aspergillus niger* isolated from decaying vegetables were cultured on MacConkey's agar (Becton Dickinson and company, Microbiology systems, sparks, MD) and nutrient agar media. Fungal cultures were prepared on Sabouraud Dextrose Agar medium (SDA).

The plant extracts obtained using methanol; diethyl ether and cold sterile distilled water were primarily screened for antibacterial activity. The agar well diffusion method (Okeke et al., 2001) was used. In this method, pure isolate of each microbe was sub cultured on the recommended specific media for each microorganism at 37°C for 24 h. From each plate four colonies were touched with a sterile loop and transferred into normal saline (0.85%) under aseptic conditions. Density of each microbial suspension was adjusted equal to that of 10.6 cfu/ml standardized by 0.5 Mc Farland standard and used as an inoculums for performing agar well diffusion assay (Aneja et al., 2010). 100 µl of inoculums of test organisms was swabbed on to the media plates. The agar plates were allowed to dry and wells or cups of 6 mm were made with a sterile borer in the Inoculated plates. The methanol and diethyl ether extracts were prepared at different dilutions (10, 5 and 2.5%) in DMSO solution. The aqueous extract was diluted in water.50 µl volume of the extracts was propelled directly into the well (in triplicate) of each culture plate containing test organisms. The plate was allowed to stand for 10 min for diffusion of extract to take place and incubated at 37°C for 24 h (Aneja et al., 2009a, b; Khokra et al., 2008; Rios et al., 1980). 50 µl volumes of the extracts were propelled directly into the well (in triplicate) of each culture plate containing test organisms. The plate were allowed to stand for 10 min for diffusion of extract to take place and incubated at 37°C for 24 h (Khokra et al., 2008). Sterile DMSO (20%) serves as negative control. Antibiotic discs of chloramphenicol (C)-(100 µg/disc) and cephoperazon (CS)-(100 µg/disc) for bacteria, and miconazole for fungus serves as positive control. MIC is the lowest concentration of compounds/extract/drug that completely inhibit the growth of the microorganisms in 24 h (Thongson et al., 2004). The inhibition zone surrounding the well containing the extracts indicates the anti-microbial activity. The diameter was measured and the experiment was performed in triplicates.

RESULTS AND DISCUSSION

Ethno botanical survey and informant consensus factor

During the survey, a total of 876 answers were obtained concerning the use of 11 medicinal plants (Table 1) which were grouped into 7 categories of medicinal uses (Figure 2 and Table 2). All these categories are more or less involved in the cure of illness of microbial origin. Among them those with the highest number of mentions were psoriasis (22.4%), diarrhoea (21.34%), scabies (19.63%), dysentery (14.20%) and cancerous wound (6%).

The group of illness of possible microbial origin that obtained the highest informant consensus factor value was that of ailments like scabies, psoriasis, dysentery, diarrhoea and cancerous wound with F_{ic} value of 0.98. The species responsible for this high consensus was *Garcinia gummi gutta* (Clusiaceae) with 60 and 75 reports, *Breynia vitis Idaea* (Phyllanthaceae) with 51 and 59 reports, *Gomphostemma heyneanum* (Lamiaceae) with 52 and 50 reports, *Nothapodytes nimmoniana (Icacinaceae)* with 48, 50 and 51 reports, *Embelia tsjeriam-cottam* with 48 reports, *Chilocarpus malabaricus (*Apocyanaceae) with 43 reports, *Chonemorpha fragrans (*Apocyanaceae) with 34 and 36 report , *Hedychium coronarium* with 20 and 25 reported events, *Raphidophora pertusa (Raphidophoraceae)* with 12 and 14 reports and *Alstonia venenata* **(**Apocynaceae) with 12 reported events. The calculation of informant consensus factor allowed a more objective selection of the species for the microbiological study in order to validate traditional knowledge (Canales et al., 2005).

Table 1. The number of use reports of 11 medicinal plants in each category.

Plant species	Disease category and number of reported events by different tribal groups						
	Dysentery	Diarrhea	Psoriasis	Scabies	Cancerous wound	Snake bite	Mumps
Alstonia venenata R.Br				12		50	
Chilocarpus malabaricus Bedd.			43.				
Chonemorpha fragrans			34	36			
Garcinia gummi gutta	60	75					
Gomphostemma heyneanum	52	50					
Raphidophora pertusa	12	14					50
Pittosporum neelgherrense						46	
Nothapodytes nimmoniana			48	50	51		
Breynia vitis Idaea			51	59			
Embelia tsjeriam-cottam		48					
Madhuka longifolia			20	15			
Total	124	187	196	172	51	96	50

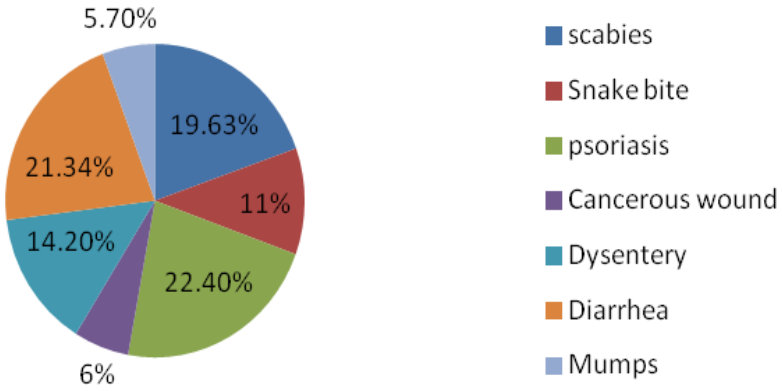

Figure 2. Seven categories of medicinal uses and their percentage.

Table 2. The seven categories of medicinal uses, total reported events and informant consensus factor.

S/N	Disease category	Total reported events	*Fic*
1	Scabies	172	0.98
2	Snake bite	96	1.
3	Psoriasis	196	0.98
4	Cancerous wound	51	1.
5	Dysentery	124	0.98
6	Diarrhea	187	0.98
7	Mumps	50	1.

Antimicrobial activity evaluation

Based on the above results, the anti bacterial and anti-fungal activities were evaluated for the 10 species. The zone of inhibition shown by these medicinal plants were tabulated and shown in Table 3. All plants have shown

Table 3. Antimicrobial activity of plant extracts.

Botanical name	*E. coli*			*Staphylococcus aureus*			*Aspergillus niger*
	Methanol extract (mm)	Diethyl ether extract (mm)	Water extract (mm)	Methanol extract (mm)	Diethyl ether extract (mm)	Water extract (mm)	Methanol extract (mm)
Alstonia venenata R.Br	8	21	-	8	15	-	-
Breynia vitis Idaea	16	-	-	10	24		-
Chilocarpus malabaricus Bedd.	-	13	-	-	16	-	-
Chonemorpha fragrans (Moon)	7	10	-	7	13	-	-
Embelia tsjeriam-cottam	14.3	9	-	7	15	-	-
Garcinia gummi gutta (L.) Robs.*var gummi-gutta*	-	-	30	-	18	-	-
Gomphostemma heyneanum Benth.*var. heyneanum*	15	13	-	9	14	-	-
Madhuka longifolia (Koenig.)J.F.Macbr	-	21	-		22	-	-
Nothapodytes nimmoniana (Graham)Mabb	15	10	-	7	15		27
Raphidophora pertusa (Roxb.)Schott.	8	11	-	6	10		-
Chloramphenicol	29			30			
Cephoperazone	8			9			
Miconazole							26

a. Inhibition zones are the mean including well diameter (6 mm); b. Antibiotic discs of chloramphenicol (C) (100 µg/disc), Cephoperazon (CS) (100 µg/disc) and Amphotericine B, Miconazole (1000 mg/ml)) are antimicrobials used as positive control. Extract quantity is 50 µl/well.

antimicrobial activity both with *E. coli* and *Staphylococcus aureus and* only *N. nimmoniana* showed antifungal activity.

The diethyl ether extracts of almost all plants focused on in the study except *Breynia vitis Idaea* showed antibacterial activity against *E. coli*. The methanolic extract of *Chilocarpus malabaricus* did not show activity against *E. coli* and *S. aureus.* The water extract of *G.gummi gutta* was found to be antibacterial against *E. coli*. The methanolic leaf extract of *N. nimmoniana* showed antifungal property against *A. niger.*

The aqueous leaf paste of *N. nimmoniana (Icacinaceae),* was used by the Kurichia, Kuruma and Kattunaika tribes of Wayanad to make herbal remedies against arthritis, cancer, skin problems like psoriasis and scabies. The results of antimicrobial activity of this species shows a MIC value of 27 mm against *A. niger*, 7 and 15 mm against *S.aureus*, 15and 10 mm against *E. coli* which give support to the plant use by these tribes. *Alstonia venenata* (MIC value 15 mm against *E. coli*) leaf paste were used by the tribes of Wayanad for the treatment of snake poison and skin diseases. The water extract of *G. gummi gutta* showed an excellent result against *E. coli* (MIC 30 mm), which is higher than the inhibition diameter of positive control, chloramphenicol (29 mm.) *G. gummi gutta* wasused by the tribes singly or in combination with other plant parts to cure various ailments. The plant *G. gummi gutta* (Clusiaceae) is a lower risk near threatened plant. The dried fruit piece is an essential ingredient in fish curries in Kerala. Its leaves were used by the Kurichia tribes of Wayanad district to prepare medicine for dysentery, diarrhea, tonsillitis, ulcer and bleeding piles. The diethyl ether extract of this plant showed an inhibition value of 18 mm against *S. aureus* which is less significant as compared to the antimicrobial results of the antibiotic chloramphenicol (29 mm).

Leaf extract of *Breynia vitis Idaea* (Phyllanthaceae) was used for curing body pain and skin problems like psoriasis and scabies. The methanolic extract of this plant showed minimum inhibition of 16 and 10 mm against *E .coli* and *S. aureus*, respectively. Diethyl ether extract of this plant showed a minimum inhibition of 24 mm against *S. aureus* . The aqueous leaf extract of *Madhuka longifolia* is mainly used by the tribes for the treating skin diseases. This extract showed inhibition with both *E. coli* (21 mm) and *S. aureus* (22 mm). The kurichia tribes use the leaves of *Gomphostemma heyneanum*, (Lamiaceae) to cure dysentery, diarrhoea and arthritis.

Table 4. Therapeutic uses of the plants selected for antimicrobial analysis.

Botanical name	Local name	Therapeutic use	Tribes associated
Alstonia venenata R.Br	Theeppala	Scabies, snake bite	Kurichia, Kuruma.
Breynia vitis Idaea	Kurukkankombu	Body pain Psoriasis, scabies,	Kurichia Kuruma Kattunaika
Chilocarpus malabaricus Bedd.	Vallippala	Skin disease	Kurichia.
Chonemorpha fragrans (Moon)	Perumkurumba.	Psoriasis, scabies, blood purification	Kurichia.
Embelia tsjeriam-cottam(Roem.&Schult.)DC	Kattuvizhal	Pneumonia, diarrhoea.	Kurichia Kuruma Kattunaika
Garcinia gummi gutta (L.) Robs.*var gummi-gutta*	Kodampuli	Tonsilites. Ulcer Bleeding piles, dysentery, diarrhoea.	Kurichia Kuruma Kattunaika Adiya Paniya
Gomphostemma heyneanum Benth.var*. heyneanum*	Theepperuku	Dysentery, diarrhoea, arthritis	Kurichia Kuruma Kattunaika
Madhuka longifolia(Koenig.)J.F.Macbr	Elippa	Psoriasis, scabies	Kuruma. Adiyan
Nothapodytes nimmoniana (Graham)Mabb	Ulukkuvetty	Cancerous wound, psoriasis, scabies, arthritis	Kurichia kurichya Kuruma Kattunaika
Pittosporum neelgherrense Wightt.	Analivenga	Snake bite	kurichya
Raphidophora pertusa(Roxb.)Schott.	Anachakkara	Dysentery, diarrhoea, mumps, tonsillitis.	Kattunaika Kurichya

Methanolic extracts of this species showed antibacterial activity against *E. coli* and *S. aureus* (Table 3). Diethyl ether extract showed activity against *E. coli* and *S. aureus* with MIC of 13 and 14 mm, respectively.

Raphidophora pertusa is a climber. Its local name is Anachakkara. Leaf juice is extensively used by the Kattunaika and Kurichia tribes for curing dysentery *and* diarrhea. In the present study, the water extract of the leaves showed no activity against *E. coli* and *S. aureus.* The diethyl ether and methanol extract showed MIC value of 10 and 6 mm against *S. aureus*, respectively. The methanolic and diethyl ether extracts against *E. coli* is less inhibitory and showed MIC values of 11 and 8 mm, respectively. The therapeutic uses of all these plants by the tribes of Wayanad district, Kerala are shown in Table 4. We isolated the tested organisms *E. coli* from stool samples of diseased persons and *S. aureus* from infected urine samples. The tribal populations of Wayanad use the plants mentioned in Table 1 to cure dysentery, diarrhea, psoriasis, wound infections and scabies. *E. coli* are bacteria that normally live in the intestines of humans and animals. Although, most strains of these bacteria are harmless, several are known to produce toxins that can cause diarrhea. The antimicrobial reports showed that the leaves of *G. gummi gutta, G. heyneanum, M. longifolia and Embelia tsjeriam-cottam* used by the tribes in Wayanad were excellent medicines for dysentery. *S. aureus* is the most common cause of staphylococcus infections. It is a spherical bacterium, frequently seen as a part of the skin flora found in the nose and on skin. About 20% of the population is long-term carriers of *S. aureus.* It can cause a wide range of illnesses like minor skin infections. The tribal people of Wayanad use *Alstonia venenata*, *Chonemorpha fragrans, Nothapodytes nimmoniana and Breynia vitis Idaea* for the treatment *of* scabies. We got good results for antimicrobial activity of these leaf extracts against these organisms. So these plant leaves can be used for the preparation of medicines against scabies like skin infections caused by *S. aureus.*

Based on the results of antimicrobial activity and ethno-medicinal values, attention has to be given for scientific bioprospection and conservation of these medicinal plants.

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Full Length Research Paper

Isolation and characterization of 1-aminocyclopropane-1-carboxylate (ACC) deaminase-containing plant growth-promoting rhizobacteria from carnation soil and roots

Haiquan Huang[1,3]*#, Meijuan Huang[1]*#, Guosheng Fan[1]*, Xuefei Liu[1], Jihua Wang[2], Qing Duan[2] and Qixiang Zhang[3]*

[1]School of Landscape Architecture, Southwest Forestry University, Kunming 650224, China.
[2]Institute of Flower Research, Yunnan Provincial Academy of Agricultural Sciences, Kunming 650205 China.
[3]School of Landscape Architecture, Beijing Forestry University, Beijing 100083, China.

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Five strains of plant growth-promoting rhizobacteria (PGPR) with 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity were isolated from carnation soil and roots using ACC as the sole nitrogen source. Based on their growth morphological, microscopic cell properties and 16S rRNA sequence analysis, the results showed that three strains were identified as *Enterobacter* section and one as *Erwinia* among four strains from the soil, and one from carnation roots wAS identified as *Acinetobacter*; there are some differences in ACC deaminase activities among all isolated strains in this study. It is suggested that ACC deaminase-containing PGPR could be a cost-effective, environment-friendly and promising potential strategy to promote plant growth, alleviate biotic and abiotic stresses and ensure sustainable agriculture, especially for ethylene-sensitive flowers production.

Key words: 1-Aminocyclopropane-1-carboxylate (ACC), deaminase, plant growth-promoting rizobacteria, carnation, salinity stress.

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are considered as advantageous bacteria in the rhizosphere, and helpful for sustainable agriculture by assisting plant growth and development directly or indirectly (Glick, 1995). Diverse mechanisms of different PGPR are reported on the basis of previous studies, including nitrogen fixation, phytohormone synthesis, mineral solubilization and phytopathogen prevention (biocontrol) (Glick and Bashan, 1997; Muhammad et al., 2007). PGPR exert some of these functions by means of specific enzymes, which agitate certain physiological and biochemical changes in plants. Among these enzymes, 1-aminocyclopropane-1-carboxylate (ACC) deaminase catalyses ACC, the immediate precursor of ethylene in higher plants, into ammonia and α-ketobutyrate (Glick et al., 1998). Ethylene is a well-known gaseous phytohormone among plant growth hormones, whose endogenous production has been correlated with various senescence processes (Abeles, 1973; Lieberman, 1979). Flowers are divided into two groups, ethylene-sensitive and ethylene-insensitive,

*Corresponding authors. E-mails: zqx@bjfu.edu.cn; 274955551@qq.com; meijuanhuang.swfu@gmail.com; haiquanhuang@gmail.com.

#These authors contributed equally to this work.

according to their productions of endogenous ethylene. A transient, rapid increase in ethylene concentration was indicated to be the cause of aging of ethylene-sensitive cut flowers (Halevy and Mayak, 1981; Woltering and van Doorn, 1988; Ludovica et al., 2011).

About half of arable lands in the world are saline (Jalili et al., 2009; Zahir et al., 2009). Yunnan province, located at the southwest part of China, is a semiarid region and the largest hub of carnation cultivation, in which most carnations are grown in the greenhouse. Because both the temperature and humidity in the greenhouse are always high, it results in high groundwater evaporation and plant transpiration and leads to salts solved in the ground water continuously moving toward the cultivation layer (De Clercq et al., 2009). Both biotic and abiotic stresses (such as salinity, heavy metals, drought and flooding) result in accelerating biosynthesis of ethylene (Kende, 1993; Johnson and Ecker, 1998) and affecting plant growth and crop production (Shao et al., 2007; Sajid et al., 2010). Carnation is economically one of the most important cut flowers cultivated for the flower market in the world. However, carnation has also been considered as a typical ethylene-sensitive flower, which suffer the saline and ethylene stresses in the greenhouse. Increasingly serious soil salinization in the cultivated layer leads to enormous losses of carnation yields and quality. Many researchers have clarified the positive effects of ACC deaminase-containing bacteria in the rhizosphere on degrading ACC to decrease or inhibit the ethylene production and alleviating different stresses on plant growth (Arshad et al., 2007; Cheng et al., 2007; Glick et al., 2007; Jalili et al., 2009; Wu et al., 2012). In this study, the bacterial strains with ACC deaminase activity from carnation cultivated soil and roots were screened and identified by using phenotypical, microscopic characters and 16S rDNA sequence analyses, which could be used as a bioinoculant to protect carnations against salinity stress environment, reduce or curb their ethylene output, promote their growth and prolong their supply time for the flower market.

MATERIALS AND METHODS

Isolation of bacteria

The soil and healthy root samples of carnation cultivated in greenhouse used for bacterial isolation were collected from several sites in Kunming, Yunnan Province, P. R. China. The bacteria were isolated using the dilution plate technique with Dworkin and Foster (DF) minimal salt medium containing $(NH_4)_2SO_4$ as the sole nitrogen source (Penrose and Glick, 2003), and ACC Dworkin and Foster minimal salt medium (ADF) with 3.0 mM ACC instead of $(NH_4)_2SO_4$ as the sole nitrogen source. The soil samples were treated by using the method of Glick et al. (2007) and roots using the method of Qin et al. (2008) with some modifications. Colonies that developed on the plates were subcultured repeatedly to acquire pure single colony, which was preserved on agar slants for further characterization and identification.

Growth curve

Five single colonies of the strains on the ADF medium plates were inoculated into the DF and ADF culture media at 28°C with vigorous shaking speed of 200 rpm, respectively. The photometric density of liquid culture at 600 nm was measured at specific intervals of time by repeating it thrice. The growth curve of different strains in different media was then drawn.

Measurement of ACC deaminase enzyme activity

Based on the initial experimental results, it was found that all the five strains could utilize ACC to grow well in ADF culture medium. The ACC deaminase activity of the strains was assayed according to the method of Penrose and Glick (2003). The calibration curve was plotted according to α-ketobutyric acid and protein concentration of cellular suspension after using toluene to treat cells. In order to draw the calibration curve of protein, bovine serum albumin (BSA) was used (Barbosa et al., 2009).

Bacterial characterization and 16S rDNA identification

The morphological and microscopic cell properties of the isolated strains were determined by using standard methods according to Bergey's Manual of Determinative Bacteriology (Holt, 1994). Besides, five bacterial strains were genetically confirmed by 16S rRNA gene sequence (Sangon Biotech (Shanghai) Co., Ltd. SongJiang Industrial Park) on the basis of comparative analysis on the GenBank database using the NCBI Blast program (Drancourt et al., 2001).

RESULTS AND DISCUSSION

It was found that carnation soil and roots samples contained several groups of bacteria when they were cultured on DF media. In order to search for some bacteria with ACC-degrading ability, carnation soil and roots were plated in DF medium without $(NH_4)_2SO_4$ supplemented with ACC as the sole nitrogen source for the isolation of ACC-degrading bacteria, meanwhile, *Bacillus subtilis* without ACC deaminase-containing activity was used as the control during all the screening procedures (Figure 1). The five colonies of ACC deaminase-containing bacteria, including four from soil (designated CS1, CS2, CS3 and CS4) and one from roots (designated CR1), were successfully screened by ADF media, respectively.

All the isolated bacteria had a marked ACC deaminase activity after incubation at 28°C, which could use ACC as a sole nitrogen substrate and catalyse it into ammonia and α-ketobutyrate. ACC deaminase activities of five different strains isolated in this study were measured and shown (Figure 2). We found that there existed much difference among the strains in the activities of ACC degradation, especially CS2 strain which had the highest level of ACC deaminase activity among the five isolated strains (Figure 2). Microscopic examination revealed that all the strains isolated in this study were Gram-negative, the cells appeared rods, and different strains had their own growth morphological characters (data not shown).

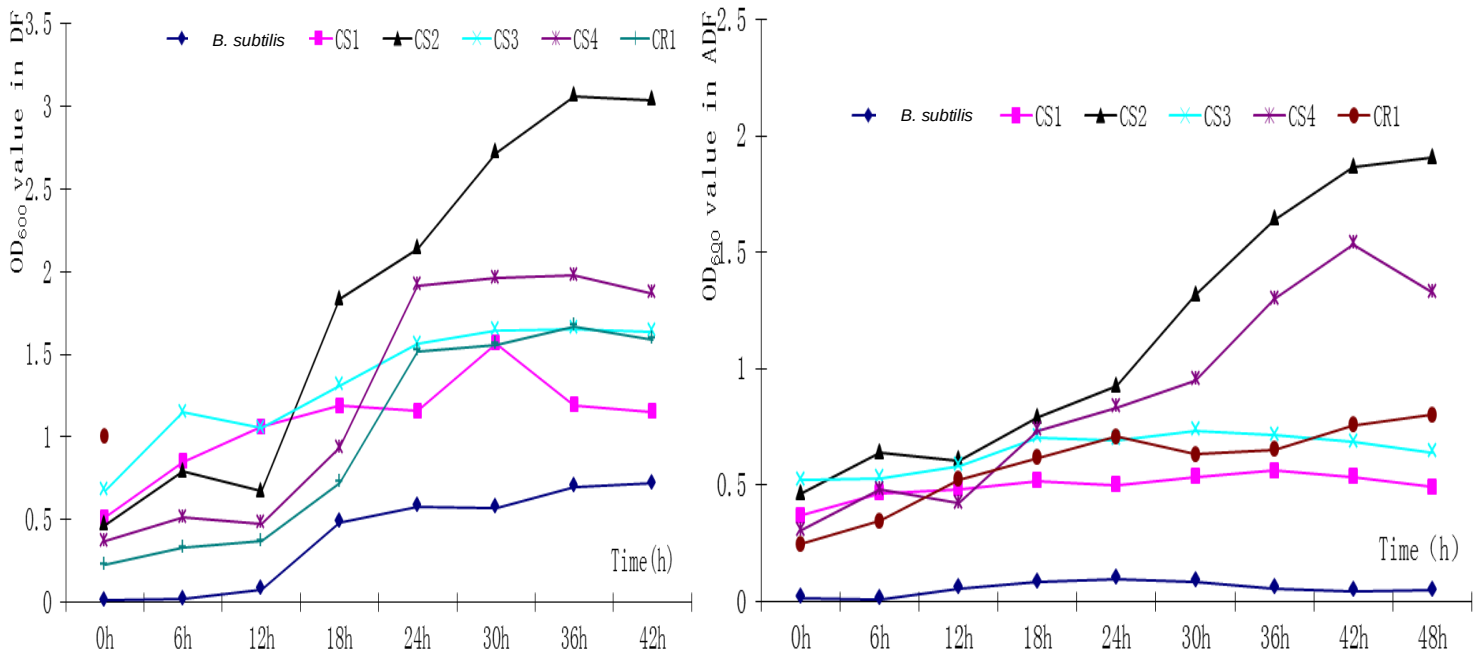

Figure 1. Growth of different isolated strains in DF and ADF culture media.

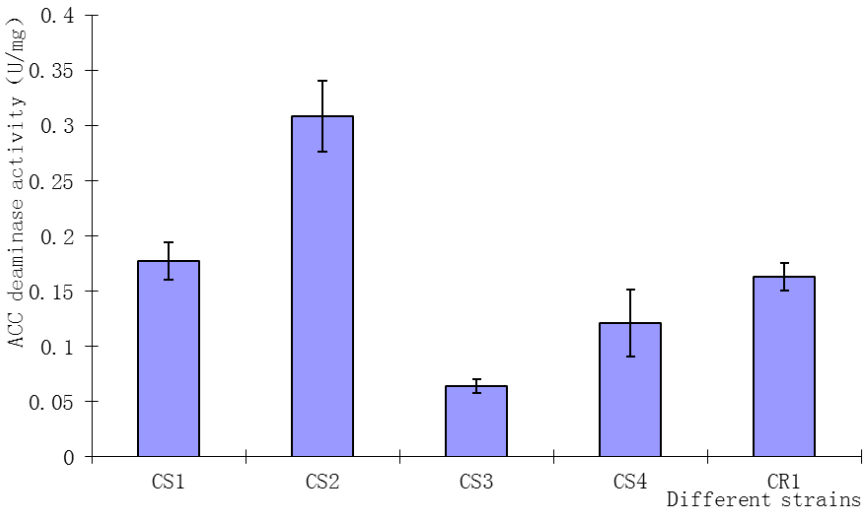

Figure 2. ACC deaminase activities of different strains isolated in this study. Error bar represents ± S.D (standard deviation) from the average of triplicates.

However, the contribution of ACC deaminase activity could be induced by ADF media. Growth of all the isolated strains was measured with the increase of turbidity at 600 nm. Figure 1 shows typical growth of the strains in the presence of $(NH_4)_2SO_4$. Growth was visualized with a perceivable lag phase and growth climax was obtained after 36 h. Relatively, weak growth occurred on ADF medium, unless it contained $(NH_4)_2SO_4$. The experiments showed that there existed some differences in the growth of different strains in ADF medium, which could be due to their different ACC deaminase activities.

The bacterial strains were consecutively confirmed by sequencing of 16S rRNA and genomic DNA analysis (Figure 3), which revealed that CS2 had 98.0% identity to the sequence of *Erwinia amylovora* (NR_041970.1), *Erwinia tasmaniensis* (NR_042422.1) and *Erwinia rhapontici* (NR_041976.1); CR1 showed 97.0% identity to the sequence of *Acinetobacter*; CS1, CS3 and CS4 possessed 99% identity to the sequence of *Enterobacter*. Thus, sequence analysis showed that different bacteria strains with ACC deaminase activity from diverse genus and family were able to degrade ACC.

Carnation, a typical ethylene-sensitive flower, produces ethylene by its autocatalytic pathway and accelerates the

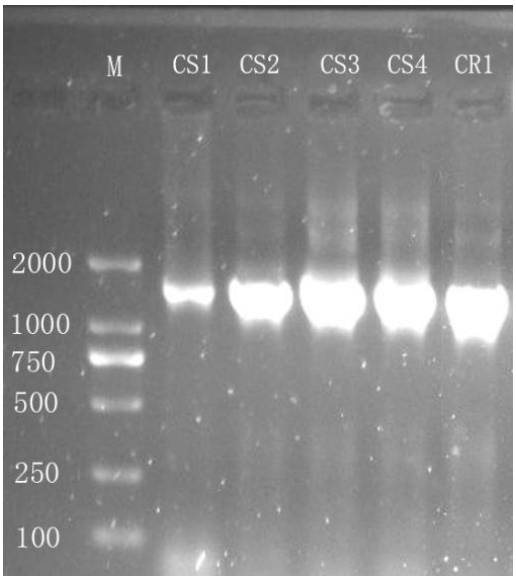

Figure 3. Agarose gel electrophoresis of PCR-amplified 16S rRNA of different isolated strains.

flower senescence (Rahemi and Jamali, 2011). Ethylene biosynthesis is influenced by many factors including inside and outside the tissues of flowers or plants. Some rhizospheric plant growth-promoting bacteria containing ACC deaminase have previously been proved to be able to slow the aging process of fresh cut carnation flower petals (Nayani et al., 1998). It has been demonstrated that ACC deaminase-containing bacterial endophytes can effectively delay the flower senescence (Ali et al., 2012). In this study, it was demonstrated that all the isolated strains had ACC deaminase activity, which were helpful to alleviate biotic and abiotic ethylene production and accumulation, and improve crop yield and quality, especially some ethylene-sensitive flowers and fruits. Furthermore, some strains may be applied as bio-inoculant for remediation of flower, fruit and crop cultivation, and their post-harvest technologies. It offers an environment-friendly and efficient method to regulate the plant ethylene for the production of some flowers and crops. More studies need to be undertaken to elucidate the mechanisms of different ACC deaminase-containing strains to promote different flower growth, strengthen their stress resistances, improve their production and quality, and extend their supply time of market and shelf life. The application of ACC deaminase-containing PGPR together with other innovations could prove to be cost-effective and environment-friendly strategy to ensure sustainable agriculture.

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African Journal of Microbiology Research

Full Length Research Paper

Virulence of new South Carolinian heterorhabditid isolates (Rhabditida: Heterorhabditidae) to the beet armyworm, *Spodoptera exigua*

R. Canhilal

Plant Protection Department, Faculty of Agriculture, Erciyes University, 38039 Melikgazi, Kayseri, Turkey.

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The virulence of new nine heterorhabditid isolates from South Carolina (*Heterorhabditis megidis* LEX, *H. zealandica* EDS and CHR, and *H. bacteriophora* WPS, SMP, PD, CFG, MF and CFM strains) against the beet armyworm was compared with two known heterorhabditid nematodes (*H. bacteriophora* Hb and HP88 strains) under laboratory conditions. The Petri-plate bioassay procedure was used to evaluate the susceptibility of the *Spodoptera exigua* larvae to the heterorhabditids at concentrations of 10, 25, 50, and 100 infective juveniles (IJs) per larva. Mortalities were counted for 4 days. At the final count, mortalities were 53.6-100, 72-100, 79.8-100, and 92.9-100% for all nematode species/strains at the concentrations of 10, 25, 50, and 100 IJs per larva, respectively. *H. megidis* LEX strain was superior and differed than others by having 100% mortality in all of the concentrations. It was second to cause early mortality. It had the highest mortality rate at 10 nematodes per larva and *H. bacteriophora* WPS, *H. zealandica* CHR, *H. bacteriophora* HP88 and *H. zealandica* EDS strains followed it with 92.9, 89.3, 85.7 and 82.1% mortality, respectively. LC_{50} for most of the nematodes was relatively low (10 IJs per larva). Virulence of *H. bacteriophora* WPS, HP88 and SMP and *H. zealandica* CHR strains were similar. The least virulent heterorhabditid was *H. bacteriophora* CFM strain with LC_{50} value of 14.8 IJs per larva. The LT_{50} value of *H. bacteriophora* WPS strain was the smallest and it was followed by *H. megidis* LEX, *H. zealandica* CHR and EDS, *H. bacteriophora* SMP, HP88, MF, PD, Hb, CFG and CFM strains, respectively.

Key words: Biological control, entomopathogenic nematodes, *Heterorhabditis, Spodoptera exigua.*

INTRODUCTION

The beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) is an important polyphagus pest of cultivated crops worldwide primarily in the tropical and subtropical regions (Stewart et al., 1996; Ansari et al., 2007; Leiva et al., 2010). It has a wide host range, including vegetable, field, and flower crops. Among susceptible vegetable crops are asparagus, bean, beet, broccoli, cabbage, cauliflower, celery, chickpea, corn, cowpea, eggplant, lettuce, onion, pea, pepper, potato, radish, spinach, sweet potato, tomato, and turnip. Field crops damaged include alfalfa, corn, cotton, peanut, safflower, sorghum, soybean, sugar beet, and tobacco (Capinera, 2006). It cannot be adequately controlled by most commercial pesticides because of its wide range of resistance (Brewer and Trumble, 1989; Van Laecke and Degheele, 1991). Its decrease in pesticide susceptibility becomes more serious, especially when it develops into late instars (Kim et al., 1998). Also, because of environmental and regulatory concerns associated with chemical use (Luckman and Metcalf, 1982; National Research Council, 1989; Hamilton et al., 1997; Cohen, 2000; Römbke et al., 2008), there is much interest in biorational approaches

E-mail: r_canhilal@hotmail.co. Tel: +90-352-437 1790. Fax : +90-352-437 6209.

(Kerns et al., 1998; Guerrero and Rosell, 2005).

Entomopathogenic nematodes (EPN) (Steinernematidae and Heterorhabditidae) are obligate parasites of insects (Poinar, 1990; Adams and Nguyen, 2002). They are mutualistically associated with bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp. for steinernematids and heterorhabditids, respectively). Infective juveniles (IJs), the only free-living stage, enter hosts through natural openings (mouth, anus, and spiracles), or in some cases, through the cuticle. After entering the host's hemocoel, nematodes release their symbiotic bacteria, which are primarily responsible for killing the host, defending against secondary invaders, and providing the nematodes with nutrition (Dowds and Peters, 2002). The nematodes molt and complete up to three generations within the host after which IJs exit the cadaver to search out new hosts (Kaya and Gaugler, 1993).

These nematodes are effective biocontrol agents of a variety of economically important insect pests (Klein, 1990; Shapiro-Ilan et al., 2002**;** Grewal et al., 2005) and they have been used in controlling insect pest for about 25 years, extending their usage from high value markets to large area crops, including forestry (Peters, 2010). A number of studies indicate that applications of EPN can result in high levels of control for a variety of noctuid pests including *S. exigua* (Feaster and Steinkraus, 1996; Medeiros et al., 2000; González-Ramírez et al., 2000; Kim et al., 2006; Kepenekçi and Evlice, 2009).

Despite the progress that has been made in the use of EPN (Laznik et al., 2010a), knowledge about their natural host range and their efficacy on insect populations as biological control agents is still limited (Ansari et al., 2007). Our overall goal was to determine the potential use of several heterordabditid nematodes for *S. exigua* suppression.

A crucial element to be successful in any biological control program with EPN is the pairing the most suitable nematode with the defined host, and relative pathogenicity among various nematodes is one of the important factors to consider in determining suitability (Georgis and Gaugler, 1991; Shapiro-Ilan et al., 2002; Shapiro et al., 2006). Therefore, this research aimed to compare the virulence of new nine heterorhabditid isolates from South Carolina against the beet armyworm with two known heterorhabditid nematodes (*Heterorhabditis bacteriophora* Hb and HP88 strains) under laboratory conditions.

MATERIALS AND METHODS

The larvae of *S. exigua* were reared on artificial pinto bean diet (Burton, 1969). *Heterorhabditis bacteriophora* Hb strain was obtained from Dr. David I. Shapiro-İlan, Integrated BioControl Systems, Inc. (Aurora, Indiana) and *H. bacteriophora* HP88 strain was provided by Dr. Khoung B. Ngyuen and Dr. Byron J. Adams of the University of Florida. The other nine heterorhabditids; *H. megidis* LEX, *H. zealandica* EDS and CHR, and *H. bacteriophora* WPS, SMP, PD, CFG, MF and CFM strains were obtained from soil on a survey in South Carolina, USA (Canhilal and Carner, 2006a).

EPN were produced on last-instar of the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) following the standard rearing method described by Woodring and Kaya (1988). A modified White Trap (Canhilal and Carner, 2006b), consisting of a folded 11-cm filter paper (3 mm in depth after folding) in a Petri dish (100 x 15mm) with 15-20 ml of distilled water, was used to collect the infective juveniles (IJs). These IJs were stored at 7-8°C in tissue culture flasks for 15-20 days before being used for experiments (Kung et al., 1990). Before the assays, viability was confirmed by observing nematode activity (rapid wiggling) under a binocular microscope (Laznik et al., 2010b).

The Petri-plate bioassay procedure was used to evaluate the susceptibility of the beet armyworm larvae to heterorhabditids at concentrations of 10, 25, 50, and 100 IJs per larva in 1 ml of sterile distilled water, following the procedures described by Woodring & Kaya (1988). Petri dishes (100 x 15 mm) were lined with two Whatman No.1 filter paper pieces (9 cm diameter). 1 h before the beginning of the experiment, the IJs were applied and distributed evenly on the filter paper. For each treatment concentration, four groups of seven 4th instars of *S. exigua* were placed per dish containing IJs. The Petri dishes were placed in a double plastic bag and put in a dark incubator at 25 ± 1°C (Glazer et al., 1991). Controls consisted of 1 ml of sterile distilled water without nematodes. The bioassay was repeated two times.

S. exigua mortality was recorded every 24 h for 4 days (Epsky and Capinera, 1994). Dead insects were incubated on modified White Traps at room temperature (25 ± 1°C) and examined to confirm the presence of nematodes. The mortalities were converted to percentages and adjusted for control mortality, using Abbott's correction formula. The data were analyzed as a completely randomized factorial design and Least Significant Difference (LSD) mean separation procedure was used to detect differences among treatments. Lethal concentration (LC_{50}) values and median lethal time (LT_{50}) values at 10, 25, 50, and 100 nematode concentrations for each nematode strain were estimated by probit analysis (SPSS, 2003).

RESULTS

All nematodes tested were capable of killing the beet armyworm and reproducing in it. The dead larvae in the treatments showed typical symptoms of nematode infection. The mortality induced by nematodes increased, typically with increasing numbers of nematodes per larva and significant positive regressions were observed between mortality and dose rate for *H. megidis* LEX (r^2 = 0.500; F = 18.000; df = 1, 19; P = 0.001), *H. zealandica* EDS (r^2 = 0.635; F = 31.281; df = 1, 19; P = 0.001) and CHR (r^2 = 0.796; F = 70.198; df = 1, 19; P = 0.001), *H. bacteriophora* HP88 (r^2 = 0.578; F = 24.672; df = 1, 19; P = 0.001), Hb (r^2 = 0.675; F = 37.402; df = 1, 19; P = 0.001), WPS (r^2 = 0.551; F = 22.120; df = 1, 19; P = 0.001), SMP (r^2 = 0.588; F = 25.657; df = 1, 19; P = 0.001), PD (r^2 = 0.730; F = 48.785; df = 1, 19; P = 0.001), CFG (r^2 = 0.670; F = 36.628; df = 1, 19; P = 0.001), MF (r^2 = 0.796; F = 70.198; df = 1, 19; P = 0.001) and CFM strains (r^2 = 0.774; F = 58.164; df = 1, 19; P = 0.001). There was very low mortality in untreated controls (Figures 1 and 2).

Low mortality occurred during the first day after treatment except 100 nematode concentrations of some

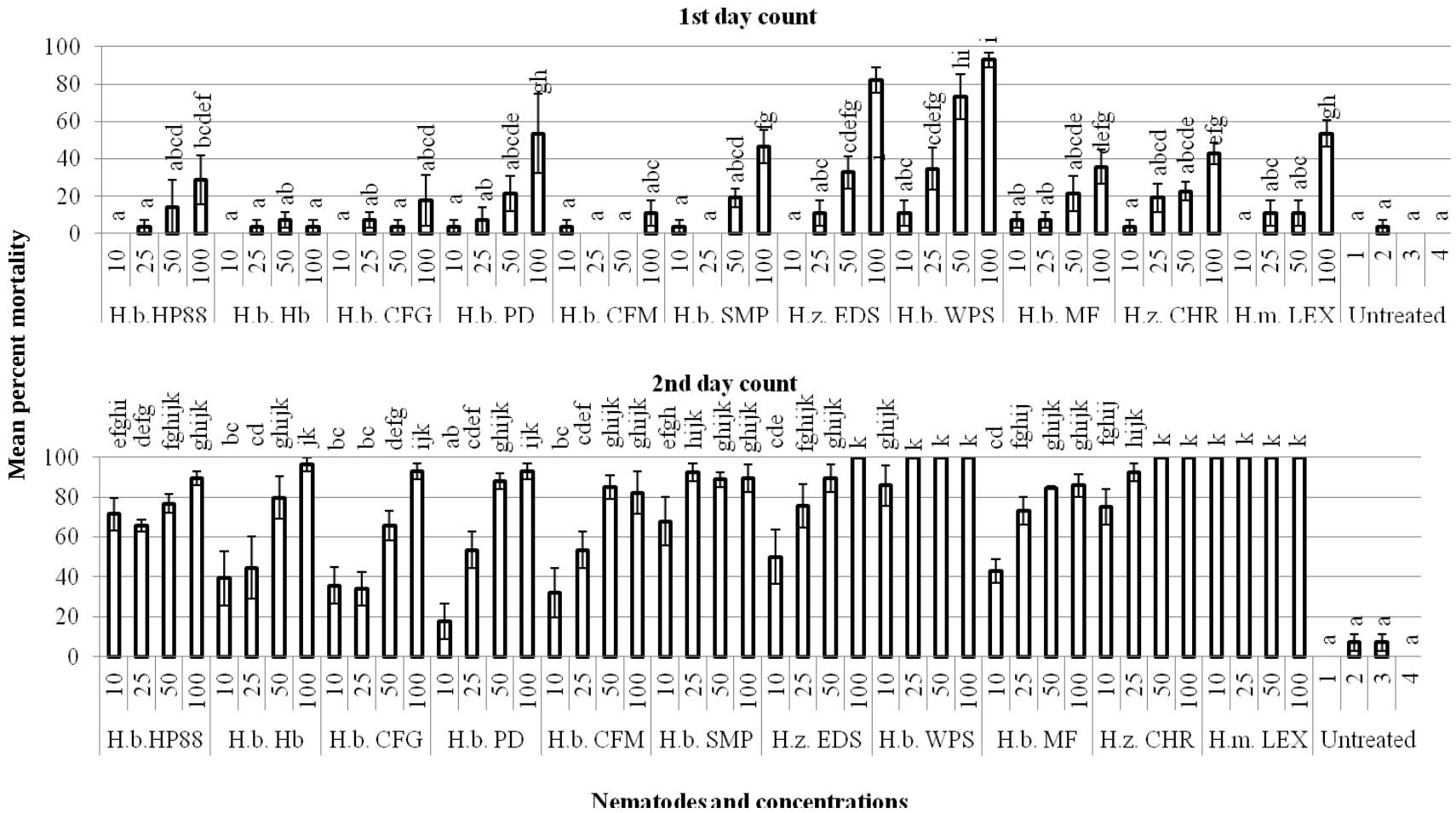

Figure 1. Mean percent mortality of 4th instars of the beet armyworm after 1 and 2 d by heterorhabditid species/strain in a Petri-plate bioassay at 10, 25, 50 and 100 IJs per larva.

nematodes and 50 nematode concentration of *H. bacteriophora* WPS strain. *H. bacteriophora* WPS and *H. zealandica EDS* strains were the best performer at the first day count (Figure 1). In general, mortality rates increased from day 2 to day 4 (Figures 1 and 2).

On the second day; mortalities at 50 and 100 nematode rates reached usually over 80%. All rates of *H. megidis* LEX strain, 50 and 100 nematode rates of *H. zealandica* CHR strain, 25, 50 and 100 nematode rates of *H. bacteriophora* WPS strain, and 100 nematode rate of *H. zealandica* EDS strain killed all larvae in the treatments (Figure 1). At 25 nematode rate; *H. megidis* LEX and *H. bacteriophora* WPS strains produced 100% mortality which were not significantly different than *H. zealandica* CHR and EDS and *H. bacteriophora* SMP strains with 92.3,

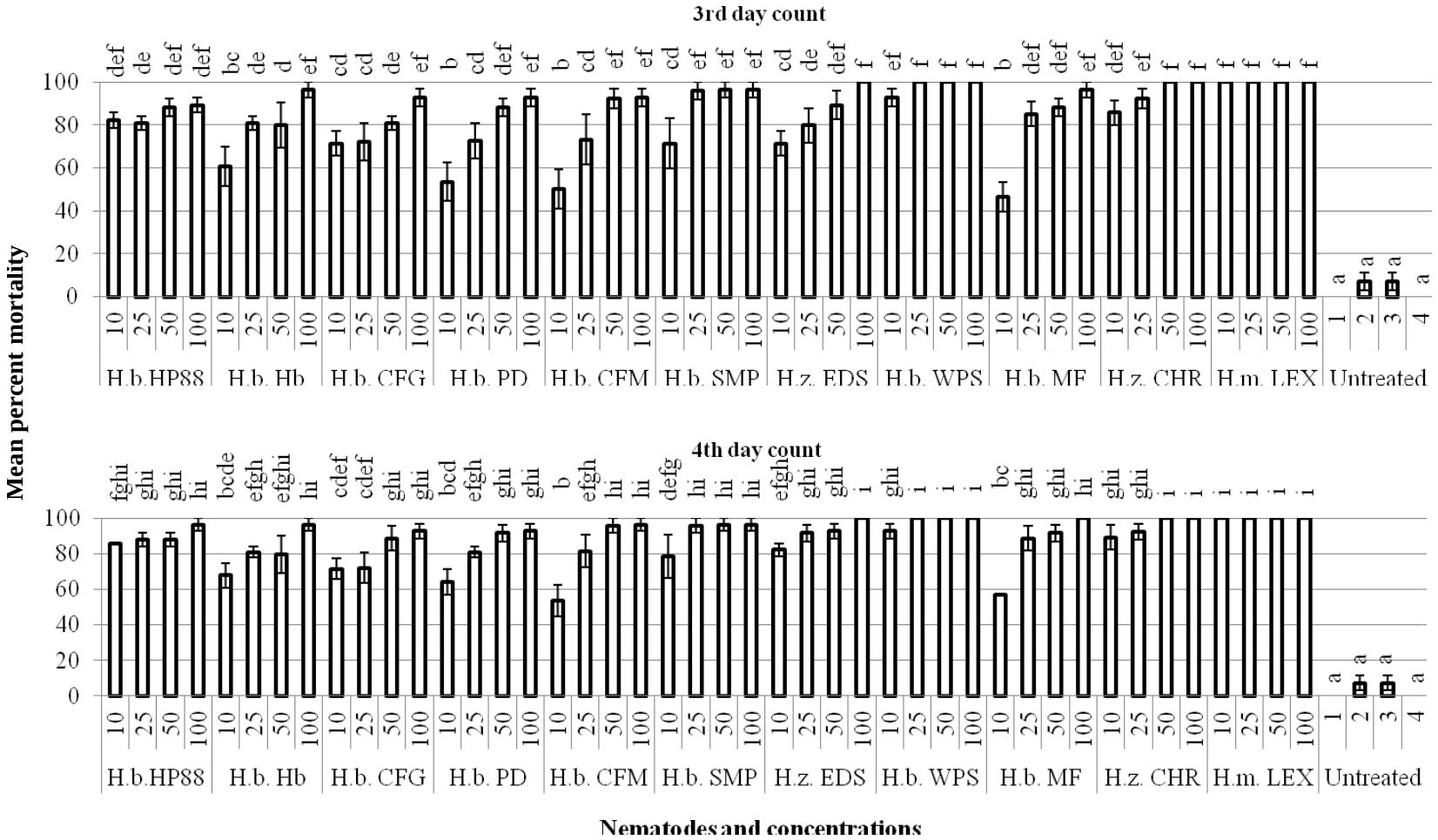

Figure 2. Mean percent mortality of 4th instars of the beet armyworm after 3 and 4 d by heterorhabditid species/strain in a Petri-plate bioassay at 10, 25, 50 and 100 IJs per larva.

75.6 and 92.3% mortality, respectively. They were followed by *H. bacteriophora* MF and HP88 strains with 73.2 and 65.5% mortality, respectively (Figure 1). There was similar trend at 10 nematode rate with 25. The lowest rate of *H. megidis* LEX strain which gave 100% mortality was not significantly different than *H. bacteriophora* WPS with 85.7% mortality. They were followed by *H. zealandica* CHR, *H. bacteriophora* HP88 and SMP, *H. zealandica* EDS strains with 75, 71.4, 67.9 and 50% mortalities, respectively. Others produced less than 50% mortality (Figure 1).

On the 3rd day count; only same strains killed all larvae in the second day caused 100% mortality

Table 1. LC_{50} and LC_{90} values of heterorhabditid nematodes for beet armyworm larvae.

Nematode	Number larvae	*LC_{50}	*LC_{90}	χ^2	df	*P*
H. megidis LEX	56	3.5	5.9	0.02	3	0.999
H. bacteriophora WPS	56	5.5	9.4	0.00	3	1.000
H. bacteriophora HP88	56	5.5	48.3	92.81	3	0.001
H. bacteriophora SMP	56	6.1	39.9	445.41	3	0.001
H. zealandica CHR	56	7.2	17.1	43.27	3	0.001
H. zealandica EDS	56	8.5	30.4	72.15	3	0.001
H. bacteriophora PD	56	12.3	59.1	143.51	3	0.001
H. bacteriophora CFG	56	12.4	63.6	59.36	3	0.001
H. bacteriophora MF	56	13.2	34.1	37.15	3	0.001
H. bacteriophora Hb	56	13.3	58.1	51.60	3	0.001
H. bacteriophora CFM	56	14.8	46.9	232.68	3	0.001

*LC_{50} and LC_{90} values were calculated over 4 rates applied and expressed in number of nematodes per larva.

(Figure 2). All nematode strains except *H. bacteriophora* HP88 produced over 90% mortality at 100 nematode rate. However they were not significantly different including *H. bacteriophora* HP88 strain.

At 50 nematode concentrations, all nematodes except *H. bacteriophora* Hb strain gave more than 80% mortality. Even it was mostly over 90% and they were not signify-cantly different except *H. bacteriophora* CFG and Hb strains. At 25 nematode rate; all nematodes strains pro-duced more than 70% mortality. It was usually over 80%. *H. megidis* LEX, *H. zealandica* CHR, *H. bacteriophora* MF, WPS and SMP strains were at the same group statistically with 100, 92.3, 85.1, 100 and 95.8% mortality, respectively (Figure 2).

At 10 nematodes rate; *H. megidis* LEX, *H. zealandica* CHR, *H. bacteriophora* WPS and HP88 strains were not significantly different with 100, 85.7, 92.9 and 82.1% mortality, respectively. *H. zealandica* EDS, *H. bacteriophora* SMP and CFG strains made other group with 71.4% mortality. Another group was *H. bacteriophora* Hb, PD, CFM and MF strains with 60.7, 53.6, 50 and 46.4% mortality (Figure 2).

At the final count, mortalities were 53.6-100, 72-100, 79.8-100, and 92.9-100% for all nematode strains at the concentrations of 10, 25, 50, and 100 IJs per larva, res-pectively. All larvae died in the treatments of *H. bacteriophora* WPS and MF, *H. zealandica* EDS and CHR, and *H. megidis* LEX strains at 100 nematode rate. Mortalities were 96.4% for *H. bacteriophora* SMP, CFM, Hb, and HP88 strains and 92.9% for *H. bacteriophora* PD and CFG strains. However, they all are not significantly different (Figure 2).

At 50 nematode concentrations, while *H. megidis* LEX, *H. zealandica* CHR, and *H. bacteriophora* WPS strains were producing 100% mortality, the others caused mortality mostly over 90%. All treatments were at the same group statistically. *H. megidis* LEX and *H. bacteriophora* WPS strains again were superior with 100% mortality at 25 IJs per larva and it was followed by *H. bacteriophora* SMP, *H. zealandica* CHR and EDS, *H. bacteriophora* HP88 an MF strains (Figure 2).

H. megidis LEX had the highest mortality rate with 100% at 10 nematode per larva. *H. bacteriophora* WPS, *H. zealandica* CHR, *H. bacteriophora* HP88 and *H. zealandica* EDS strains followed it with 92.9, 89.3, 85.7 and 82.1% mortality, respectively at same statistical group (Figure 2).

LC_{50} for most of the nematodes was relatively low (<10 IJs per larva). The LC_{50} and LC_{90} data are summarized in Table 1. The lowest LC_{50} value was obtained by *H. megidis* LEX strain (3.5 IJs per larva). Virulence of *H. bacteriophora* WPS, HP88 and SMP and *H. zealandica* CHR strains were similar (LC_{50}s ranging from 5.5 IJs per larva in *H. bacteriophora* WPS strain to 7.2 IJs per larva in *H. zealandica* CHR strain). The least virulent heterorhabditid was *H. bacteriophora* CFM strain with LC_{50} value of 14.8 IJs per beet armyworm larva.

The LT_{50} and LT_{90} data are given in Table 2. The LT_{50} values ranged from 1.5 to 3.4 days for *H. megidis* LEX and *H. bacteriophora* CFM strains at 10 IJS per larva, from 1.1 to 2.6 days for *H. bacteriophora* WPS and *H. bacteriophora* CFG and CFM strains at 25 IJs per larva, from 0.8 to 2.1 days for *H. bacteriophora* WPS and CFG strains at 50 IJs per larva, and from 0.6 to 1.7 days for *H. bacteriophora* WPS and CFM strains at 100 IJs per larva, respectively. The LT_{50} value of *H. bacteriophora* WPS strain was the smallest and it was followed by *H. megidis* LEX, *H. zealandica* CHR and EDS, *H. bacteriophora* SMP, HP88, MF, PD, Hb, CFG and CFM strains, respectively when the average of LT_{50} values at four concentrations were evaluated.

DISCUSSION

In determining an entomopathogenic nematode as a

Table 2. LT_{50} and LT_{90} values of heterorhabditid nematodes at 10, 25, 50, and 100 infective juveniles per larva for beet armyworm.

Nem[a]	Number of larvae	10 infective juveniles				25 infective juveniles			
		*LT_{50}	*LT_{90}	χ^2	P	LT_{50}	LT_{90}	χ^2	P
HP88	56	1.5	1.8	1.16	0.763	1.3	1.6	0.09	0.994
Hb	56	1.7	2.9	50.89	0.001	1.1	1.5	0.08	0.994
CFG	56	2.0	3.3	37.10	0.001	1.6	2.8	56.44	0.001
PD	56	2.2	3.6	41.08	0.001	2.1	3.5	26.37	0.001
CFM	56	2.3	4.0	35.40	0.001	1.7	2.6	284.97	0.001
SMP	56	2.5	4.0	19.42	0.001	1.8	3.2	23.87	0.001
EDS	56	2.8	4.4	18.96	0.001	2.6	4.3	12.82	0.005
WPS	56	2.9	4.7	17.45	0.001	2.4	3.8	20.32	0.001
MF	56	3.2	5.6	14.50	0.002	2.0	3.3	28.48	0.001
CHR	56	3.2	5.0	4.62	0.202	2.3	3.9	17.31	0.001
LEX	56	3.4	5.6	11.63	0.009	2.6	4.1	18.33	0.001
		50 infective juveniles				**100 infective juveniles**			
WPS	56	0.8	1.6	0.11	0.991	0.6	1.3	0.36	0.949
CHR	56	1.2	1.2	0.01	1.000	1.1	0.9	0.05	0.997
LEX	56	1.3	1.5	0.09	0.994	0.9	1.4	0.05	0.997
EDS	56	1.4	3.2	40.82	0.001	0.8	2.8	0.17	0.982
SMP	56	1.5	2.6	69.37	0.001	1.3	2.4	51.74	0.001
PD	56	1.6	2.8	44.86	0.001	1.2	1.1	50.29	0.001
MF	56	1.7	3.5	32.34	0.001	1.3	2.8	1.90	0.593
HP88	56	1.8	3.6	33.67	0.001	1.5	2.5	26.40	0.001
CFM	56	1.8	3.0	78.17	0.001	1.7	2.1	34.52	0.001
Hb	56	2.0	2.9	49.73	0.001	1.6	2.7	288.01	0.001
CFG	56	2.1	2.7	26.37	0.001	1.6	2.8	59.90	0.001

[a]Nem= Nematodes; WPS= *H. bacteriophora* WPS; CHR= *H. zealandica* CHR; LEX= *H. megidis* LEX; EDS= *H. zealandica* EDS; SMP= *H. bacteriophora* SMP; PD= *H. bacteriophora* PD; MF= *H. bacteriophora* MF; HP88= *H. bacteriophora* HP88; CFM= *H. bacteriophora* CFM; Hb= *H. bacteriophora* Hb; CFG= *H. bacteriophora* CFG. *LT_{50} and LT_{90} values were calculated over 4 days counts and expressed in days.

biological control agent, it is important to look at several attributes of the agent such as attraction, penetration, movement, host defense mechanisms, and biotic and abiotic environmental factors. Although many factors are responsible for the level of infectivity, some basic data may be gathered through lab studies (Mannion and Jansson, 1992; Shapiro-Ilan et al., 2002; Laznik et al., 2011).

The virulence of nematodes to noctuids and other lepidopters varies significantly (Mbata and Shapiro-Ilan, 2005; Mederios et al., 2000). Mederios et al. (2000) reported that *H. bacteriophora* Az33 strain caused 23% mortality to the sixth instar of another noctuid, *Pseudaletia unipuncta*, the armyworm at 200 IJs per larva, whereas, the mortality of *H. bacteriophora* strains in our research was much higher even at lower rates. This may be because of different host and difference of strain which was collected from different locality.

Some heterorhabditid nematodes may possess additional positive attributes compared with others as demonstrated with ranging percent mortalities on large scales (53.6-100%) in the current study. Although the beet army worm larva was susceptible to each nematode species and strain tested, there were differences among these nematodes in their ability to kill the insect. *H. megidis* LEX, *H. bacteriophora* WPS, HP88 and SMP, *H. zealandica* CHR and EDS strains were more efficacious than others against *S. exigua* larva as it was reflected in the LC_{50}, LT_{50} and percent mortality data. Mortalities were higher, LC_{50} values were lower and LT_{50} values were shorter for these nematodes. Kim et al. (2006) reported that LC_{50} value of *H. bacteriophora* Hamyang strain was 5.5 IJs per 4th instar of the beet armyworm. This value is the same as LC_{50}s of *H. bacteriophora* WPS and HP88 strains (5.5 IJs per larva) and close to *H. bacteriophora* SMP strain (6.1 IJs per larva) in our study. On the other hand, it was quite different than LC_{50}s of other *H. bacteriophora* strains which ranged from 12.3 to 14.8 IJs per larva. These differences may be due to difference of the origins of the strains (Mannion and Jansson, 1992).

No statistical difference was obtained among nematode strains at 50 and 100 nematode concentrations. Therefore

10 and 25 nematode per larva were distinctive rates to differentiate the nematodes' biological efficacy on the beet armyworm. *H. megidis* LEX strain was superior and differed than others by having 100% mortality in all of concentrations and it was second to cause early mortality.

All the heterorhabditid strains tested showed high virulence to 4th instars of the beet armyworm, producing a significantly higher mortality (53.6-100%) at all concentrations than the untreated control at the final count. Apparently, they are effective bio-control agents of *S. exigua* and our results corroborate the finding of the study of Kim et al. (2006). However, environmental factors such as soil structure, temperature, humidity and host density under greenhouse and field conditions have huge impact on the efficacy of EPN (Koppenhöfer, 2000; Georgis et al, 2006). Therefore future studies need be directed to the greenhouse and field conditions with these heterorhabditid isolates.

Our results suggest that *H. megidis* LEX strain, *H. bacteriophora* WPS, SMP and HP88 strains, and *H. zealandica* EDS and CHR strains should be consider first to be studied further as potential biocontrol agents of the beet armyworm and other similar lepidopters. The others, *H. bacteriophora* PD, CFG, MF, Hb and CFM strains may also be valuable material to be studied.

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Full Length Research Paper

L-Asparaginase produced by Streptomyces strain isolated from Egyptian soil: Purification, characterization and evaluation of its anti-tumor

Sabha Mahmoud El-Sabbagh[1], Nadia Hamed El-Batanony[2*] and Tarek A.Salem[3]

[1]Botany Department, Faculty of Science, Menoufiya University, Shebin El-Kom, Menoufiya Governorate, Egypt.
[2]Department of Sustainable Development,Environmental Studies and Research Institute (ESRI) , University of Sadat City, P. O. 32897 Sadat City, Menoufiya Governorate, Egypt.
[3]Department of Biochemistry, College of Medicine, Qassim University, Kingdom of South Africa (KSA).

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L-Asparaginase (EC 3.5.1.1) is produced from actinomycetes to avoid the hypersensitive effect of that produced from other bacteria. *Streptomyces halstedii* strain was isolated from Egyptian soil and produced L-asparaginase. The 55.2-fold purified enzyme obtained had a final specific activity of 2071.2 U/mg. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed one band with molecular weight of 100 KDa. The K_m value was 0.1939 mM. The enzyme showed maximum activity at pH 8.0, at optimum temperature at 37°C. Ethylene diamine tetraacetic acid (EDTA) and metal ions such as Zn^{2+}, Hg^{2+} Cu^{2+} and K^{+} decreased the activity of the enzyme. Ions such as Ca^{2+} and Fe^{2+} did not affect the activity of the enzyme. The enzyme showed cytotoxic activity against Ehrlich ascites cells (EAC) *in vitro*. *In vivo,* it showed a significant reduction in malondialdehyde (MDA) levels as the end product of lipid peroxidation and a remarkable increase in activity of liver antioxidant enzymes, [superoxide dismutase (SOD), catalase (CAT)] and a reduction in tumor weight. In conclusion, L-asparaginase from *S. halstedii* showed anti-tumor activity and cytotoxic effect against cancer cell line *in vitro* and *in vitro*. The reduction of tumor size in albino mice may be attributed to the elevation of CAT and SOD activities as well as the diminishing of MDA.

Key words: L-Asparaginase purification, antitumour activity, *Streptomyces* sp.

INTRODUCTION

L-Asparaginase (EC 3.5.1.1) is the first enzyme with anti-tumor activity intensively studied in human beings (Savitri and Azmi, 2003). It is used for the treatment of malignancies of the multiorgans (Kumar and Selvam, 2011). It is used widely as a therapeutic agent for treating acute lymphoblastic leukemia in children and lympho-sarcoma (Khamna et al., 2009).

The amino acid, L-aspargine, is essentially required for the survival of both normal and cancer cells. Most normal tissue synthesizes L-asparagine in amounts sufficient for their metabolic needs with their own enzyme, L-asparagine synthetase. However, certain malignant cells cannot synthesize L-asparagine and must consequently rely on an external supply in the plasma and tissues. Since several types of tumor cells require L-asparagine for protein synthesis, they are deprived of an essential growth factor in the presence of L-asparaginase. The administration of this enzyme can digest its substrate resulting in starving and killing certain cancer cells (Basha et al., 2009; Kumar and Selvam, 2011). This fact suggested the development of this enzyme as a potent anti-tumor or anti-leukemic drug (Savitri and Azmi, 2003).

Since extraction of L-asparaginase from mammalian cells is difficult, microorganisms have proved to be a

*Corresponding author. E-mail: nelbatanony@yahoo.com. Tel: 00201095171515. Fax: 0020482600404.

better alternative for L-asparaginase extraction, thus facilitating its large scale production (Sahu et al., 2007). Various bacterial sources such as *Escherichia coli*, *Staphylococcus* sp., *Pseudomonas aeruginosa* (Moorthy et al., 2010), *Thermus thermophilus* (Pritsa et al., 2001), *T. aquaticus* (Curran et al., 1985), *Enterobacter aerogenes* (Mukherjee et al., 2000), *Zymomonas mobilis* (Pinheiro et al., 2001) and *Bacillus licheniformis* (Golden and Bernlohr 1985) produce L-asparaginase. However, L-asparaginase from bacterial sources causes hypersensitivity in the long-term use, leading to allergic reactions and anaphylaxis (Sahu et al., 2007).

The search for other L-asparaginase sources, like actinomycetes, can lead to an enzyme with less adverse effects. Actinomycetes represent a good source for the production of L-asparaginase (Savitri and Azmi, 2003). The production of L-asparaginase has been studied in *Streptomyces griseus, S. Karnatakensis, S. albidoflavus, S. gulbargensis* (Kattimani et al., 2009), *S. aurantiacus* (Gupta et al., 2007), *S. aureofasculus, S. canus, S. olivoviridis* (Sahu et al., 2007), a marine *Streptomyces* sp. PDK2 (Narayana et al., 2008) and *Nocardia* sp. (Gunasekaran et al., 1995).

This work aimed to purify, to characterize and to assess the potential anti-tumor activity of L-asparaginase from *S. halstedii* isolated from Egyptian soil.

MATERIALS AND METHODS

Isolation of actinomycetes

Soil samples were collected from different localities of Menoufiya governorate located in the Northern of Egypt. Thirty (30) isolates of actinomycetes were isolated from soil by the dilution plate method on starch-nitrate agar plates (Waksman and Lechevalier, 1962). The plates were incubated for seven days at 30°C. Colonies were checked for purity by repeated sub-culturing and the pure colonies maintained on slants of the same medium and stored at 4°C.

Screening for L-asparaginase production

All actinomycetes strains were evaluated for their ability to produce L-asparaginase according to the procedure of Khamna et al. (2009). Each strain of actinomycetes was inoculated on glycerol asparagine agar (Pridham and Lyons, 1961) incorporated with pH indicator; pH was adjusted to 7.0 and incubated at 30°C for seven days. Colonies with pink zones were considered as L-asparaginase-producing active strains. Two control plates were also prepared using glycerol asparagine agar; one was without dye while the other was without asparagine. The more potent strains were selected for fermentation process.

Fermentation procedure

Fermentation was carried out for the active actinomycetes strains, using 250 ml capacity Erlenmeyer flasks, containing 50 ml of glycerol asparagine medium. Each flask was inoculated by 1 ml of spore suspension ($2.0x10^6$ spores /ml) of three days old culture. Inoculated flasks were incubated at 30ºC for five days on a rotary shaking incubator at 250 rpm. Samples were taken periodically every day for determination of L- asparaginase activity.

Determination of L-asparaginase activity

The activity of produced L-asparaginase was assessed according to the method of Mashburn and Wriston (1963). A mixture of 0.1 ml of enzyme extract, 0.2 ml of 0.05 M Tris-HCl buffer (pH 8.6), and 1.7 ml of 0.01 M L-asparagine was incubated for 10 min at 37°C. The reaction was stopped by the addition of 0.5 ml of 1.5 M trichloroacetic acid. The ammonia released in the supernatant was determined colorimetrically at wavelength of 480 nm.

Purification of L-asparaginase with HPLC system using sephadex G-200

Ammonium sulfate was added to the crude extract from cultures grown on glycerol asparagine broth medium at 4°C to purify the L-asparaginase. The L-asparaginase activity was associated with the fraction precipitated at 70% saturation. The precipitate was collected by centrifugation at 10,000 rpm for 20 min, dissolved in 50 mM Tris-HCl buffer pH 8.6 and dialyzed against the same buffer. Using HPLC system, the concentrated enzyme solution was applied to the column of sephadex G-200 (1.5×45 cm) (Pharmacia fine co., Uppsala, Sweden) that was pre-equilibrated with 50 mM Tris-HCl buffer pH 8.6. The protein elution was done with the same buffer at a flow rate of 3 ml/30 min. It was eluted with NaCl gradient (0.1-0.5 M) and 0.1 M borate buffer at pH 7.0. The active fractions were collected, dialyzed and concentrated. The protein content was estimated by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

SDS-polyacrylamide gel electrophoresis

The molecular weight of the purified L-asparaginase sample was determined by using 10% SDS-PAGE according to the method of Laemmli (1970).

Characterization of the purified L-asparaginase

The independent parameters were evaluated at a time keeping other parameters constant. The optimized parameter obtained (based on highest enzyme activity achieved) was incorporated in the next experiment while optimizing the next parameters.

Optimum pH for L- asparaginase activity

Fixed volume of 0.1 ml of the purified enzyme solution was mixed with 0.2 ml of 0.05 M buffers of different pH from 5 to 11. Potassium phosphate was used for pH range of 5 to 7, Tris-HCl was used for pH range of 8 to 9 and glycine-NaOH was used for pH range of 10 to 11, respectively. The optimum pH for L-asparaginase activity was studied by the method of Mashburn and Wriston (1963).

Optimum temperature for L-asparaginase activity and stability

Optimum temperature for the enzyme activity was determined by incubating the assay mixture for 30 min at temperatures ranging from 20 to 70°C. After the end of the incubation periods, enzyme activity was measured as previously mentioned in optimum pH for L-asparaginase activity.

To study the optimum temperature for L-asparaginase stability, only modification of incubation temperature, incubation time and

substrate of the enzyme were made. The solution mixtures of 0.1 ml of enzyme solution and 0.1 ml of 0.05 M Tris-HCl buffer pH 8 was incubated for 15, 30, 45, 60, 90, 120, 150 and 180 min at different degrees of temperature varying from 30 to 70°C in 10°C increments and was directly cooled in ice bath. Enzyme activity of each tube was measured as previously described (Aljewari et al., 2010).

Effects of metal ions and some inhibitors on L-asparaginase activity

To determine the effect of different metal ions (K^+, Ca^{+2}, Fe^{+2}, Zn^{+2}, Cu^{+2}, Hg^{+2} and EDTA), different salt solutions ($KMnO_4$, $CaCl_2$, $FeSO_4$, $ZnCl_2$, $Cu(C_2H_3O_2)_2$, $Hg(C_2H_3O_2)_2$ and EDTA were added to the enzyme-substrate reaction mixture to yield final concentrations of 0.25, 0.5, 1, 5, and 10 mM. The mixtures were incubated for 30 min. and L-asparaginase specific activity of each sample was then determined.

Kinetics of the purified enzyme

To measure the kinetics of L-asparaginase, Michaelis constant (K_m), and maximal velocity (V_{max}) of the purified enzyme was determined. They are one of the important parameters for the evaluation of the potential usefulness of the enzyme for anti-leukaemic therapy. They were determined using L-asparagine as substrate in the range of 0.2 to 4.0 mM. Each reported reaction velocity is the mean of at least three measurements. The apparent K_m was calculated from the double-reciprocal Lineweaver-Burk plot (Basha et al., 2009).

Determination of anti-tumor property of the enzyme

In-vitro assessment of anti-tumor activity

The anti-tumor activity of the purified L-asparaginase preparations was determined *in vitro* against Ehrlich ascites carcinoma (EAC); a cell line which was kindly provided by the National Cancer Institute, Cairo, Egypt. Briefly, EAC cells were suspended in RPMI-1640 complete medium (each 100 ml contains 10% fetal bovine serum, 10 µg/ml streptomycin and 100 U/ml penicillin) and seeded in 96 flat-bottomed wells plate at a concentration of $2x10^6$/well. 200 µL of different L-asparaginase preparations (10, 20 and 50 U/ml) were seeded in triplicates and then incubated for 24 h in 5% CO_2 incubator at 37°C (El-khawaga et al., 2003). Cells viability was checked by using trypan blue staining and cytotoxicity values were calculated (Boyum, 1967).

In-vivo anti-tumor activity

Female Albino mice with body weight of 20 to 22 g were injected with $2x10^5$ EAC cells subcutaneously (S.C.) between thighs to induce solid tumor according to Mohamed et al. (2003). Animals were randomly divided into three groups (8 mice each). 24 h after EAC inoculation, one group of animals was intraperitoneal (i.p.) injected with 0.2 ml of 50 U/ml L- asparaginase preparation for five consecutive days. Another group of animals was intraperitoneally treated with 0.2 ml of anticancer drug thalidomide (1.25 mM/kg) for five consecutive days and served as control group. The tumor positive control group was treated with 0.2 ml of 0.9% normal saline. After three weeks from EAC implantation, animals were sacrificed and solid tumors were excised and weighed. Also, livers were removed, rinsed with 0.9% physiological saline and kept at -20°C and used for lipid perioxidation determination and assessment of the activity of antioxidant enzymes (CAT and SOD).

Preparation of liver homogenate

A liver tissue of 0.5 g was cut into small pieces and homogenized in 5 mL of cold phosphate buffered saline, pH 7.2, by using mechanical homogenizer. Liver homogenate was centrifuged at 8000 rpm at 4°C for 5 min, and then, the supernatant was transferred into small aliquots and kept at -70°C.

Determination of antioxidant enzymes activity in liver homogenate

Hepatic CAT activity was determined according to the method described by Slaughter and O'Brien (2000). Briefly, the reminder H_2O_2 was assayed spectrophotometrically in the presence of a chromogen at 560 nm after reaction of the catalase enzyme with definite amount of H_2O_2. Results were expressed as U/g tissue. Meanwhile, hepatic SOD activity was assayed according to the method described by Rest and Spitznagel (1977). This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye, the produced color measured at 560 nm. Results were expressed as U/g tissue.

Determination of hepatic malondialdehyde (MDA) level

Malondialdehyde (MDA) as the end product of lipid peroxidation was estimated in liver homogenate according to the procedure of Ohkawa et al. (1979). The reaction mixture contained 0.1 ml sample, 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic acid, and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid. The pH of the mixture was adjusted to 3.5 and the volume was finally made up to 4 ml with distilled water and 5 ml of the mixture of n-butanol and pyridine (15:1, v/v) was added. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the absorbance of the organic layer was measured at 532 nm. Results were expressed as nmol/g tissue.

Statistical analysis

The statistical significance of the experimental biochemical results was determined by the Student's t-test Murray (1982). For all analyses, $p<0.05$ was accepted as a significant probability level.

RESULTS

Isolation of actinomycetes and L-asparaginase activity

Preliminarily screening showed that six strains of actinomycetes exhibited L-asparaginase activity. All active strains were cultivated in glycerol-asparagine broth. Production of L-asparaginase began after 72 h of cultivation (1.3 U/ml) and reached to maximum level (3.9 U/ml) after 120 h of incubation. Out of the six active strains, *S. halstedii* (data of identification not showed here) showed highest L-asparaginase activity (8.643 µmol ammonia/ml) after 120 h of incubation.

Table 1. Effect of different concentrations of ammonium sulfate on L-asparaginase activity, protein content and specific activity.

Concentration of ammonium sulfate%	Activity (U)	Protein content (mg)	Specific activity (U/mg protein)
40	9.88 ±0.35	0.62 ±0.03	15.93 ±0.02
50	19.10 ±0.26	0.76 ±0.03	25.13 ±0.35
60	22.36 ±0.33	0.82 ±0.03	27.27 ±0.24
70	40.97 ±1.08	0.93 ±0.02	44.05 ±0.16
80	37.29 ±0.6	1.45 ±0.06	25.72 ±0.09
90	33.47 ±1.01	1.87 ±0.04	17.9 ±0.4

Purification of L-asparaginase

Optimization of ammonium sulfate precipitation

The results presented in Table 1 show that as ammonium sulfate concentration increased, L-asparaginase activity and the specific activity in the precipitates increased. They reached their maximum value at 70% saturation, then, they decreased by increasing ammonium sulfate concentration above 70%. So the 70% saturation was selected for the first step in the purification process of the enzyme.

Purification of L-asparaginase with HPLC system using sephadex G-200

Purification of L-asparaginase was carried out in three steps as shown in Table 2. Purification of L-asparaginase crude extract as affected by ammonium sulfate (70%) precipitation showed that, most of the enzyme activity was preserved in the precipitate. Total protein decreased from 2.3 to 1.86 mg, and specific activity increased from 37.5 to 228.7I U/mg protein, at approximately 6.09 folds purity in the ammonium sulfate precipitation step.

Precipitated enzyme sample was further purified with sephadex G-200 using HPLC system. Figure 1 shows that active fractionations started from fractions 14 -18. HPLC system purification step resulted in 2071.3 fold increase in specific activity of L-asparaginase produced by *S. halstedii* and the purification fold was also increased to 55.2 (Table 2). All purification steps produced an increase in specific activity.

Determination of protein molecular weight of the purified enzyme

Figure 2 shows the protein profile analysed by 10% SDS-PAGE. Analysis of the gel revealed that there was no detectable contamination as it represented just one distinct band and the molecular weight of the band of 100 kDa.

Characterization of the purified L-asparaginase

Optimum pH

The results of L-asparaginase activity in different pH values showed that the enzyme has quite a wide range of activity between 5 and 10 (Figure 3). At pH below 5 and above 10, the enzyme activity was very low. At pH around 6 and 9, the activity drops to about 60% of its maximum. Also, according to Figure 3, the profile of enzyme activity showed that there was an increase in the enzyme activity as pH increased until it reaches its optimal activity at pH 8.

Optimum temperature for L-asparaginase activity and stability

Optimum temperature of L-asparaginase is given in Figure 4. It showed that the enzyme was active at a wide range of temperature condition from 20 to 70°C. The optimum temperature for L-asparaginase activity was found to be 37°C. At higher temperatures, the reaction rate declined sharply.

Thermal stability behavior of L-asparaginase as a function of heating time is shown in Figure 5. The data indicated that no significant enzyme activity was lost when it was pre incubated at 30 and 40°C for 180 min. At 50ºC, about 80% of the enzyme activity was retained at 150 min incubation time. The enzyme lost about 50% of its residual activity when it was pre incubated at 60°C for 150 min.while at 70°C the enzyme lost about 50% at 45 min incubation.

Effects of some inhibitors and metal ions on the activity of the purified L-asparaginase enzyme

Several concentrations of EDTA (0.25-10 mM) were used to check its effect on the activity of the purified enzyme. The data of Table 3 shows that as the concentration of EDTA increases, the activity gradually decreases; at 10 mM of EDTA, the enzyme retained 52% of its activity. L-

Table 2. Purification profile of L-asparaginase from *S. halstedii.*

Purification step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification folds	Yield (%)
Crude extract	100	86.4 ±1.41	2.3 ±0.28	37.56 ±5028	1.0 ±0	100.0 ±0
Ammonium sulfate precipitation (70%)	10	425.4 ±1.64	1.86 ±0.04	228.71 ±4.04	6.02 ±0.11	492.36 ±1.9
Sephadex G-200 filtration	3	360.4 ±1.51	0.174 ±0.004	2071.26 ±56.35	54.52 ±1.49	417.1±1.75

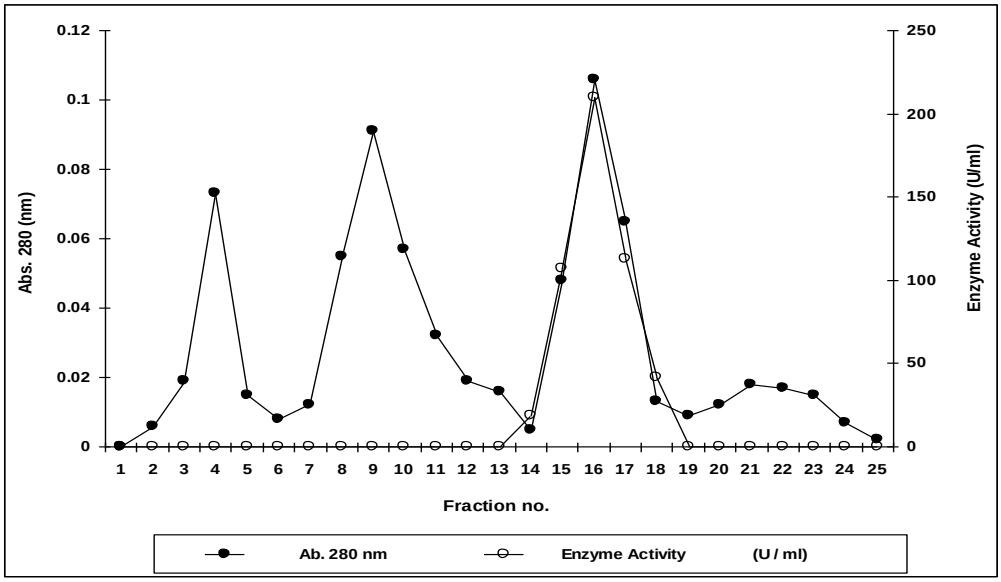

Figure 1. Elution profile of preparations obtained from *S. halstedii* by ammonium sulphate (70%saturation) using Sephadex G-200 gel filtration chromatography.

asparaginase activity was assayed in the presence of various ions and reagents (Table 3). Among the tested ions, Ca^{+2} and Fe^{+2} acted as enzyme inducer except at concentration of 10 mM of $FeSO_4$, it retained 91% of its activity. However, the enzyme retained about 50% of its activity when Zn^{2+} and Cu^{2+} ions were used at 10 mM concentration as well as EDTA. Moreover, the enzyme retained about 20 and 36% of its activity when 10 mM of Hg^{++} and K^{+} were used, respectively.

Kinetic parameter

The K_m and V_{max} of purified L-asparaginase from *S. halstedii* were 0.1939 mM and 1.22 mM/min, respectively (Figure 6).

In-vitro cytotoxic effect of L-asparaginase

As indicated in Table 4, there was a gradual decrease in the viability of EAC cell with increasing doses of the purified L-asparaginase *in vitro*. As the concentration of L-asparaginase increased, the rate of cell proliferation decreased as a result of increased inhibition efficiency on cell growth.

Effect of the purified L-asparaginase on growth of solid tumor

Table 5 shows the effect of the purified L- asparaginase on the growth of solid tumor induced in female Albino mice. The results show a significant reduction in tumor weight as compared to that of tumor control group. Moreover, L-asparaginase exhibit potent inhibitory action on the tumor growth as compared to that of thalidomide anti-tumor drug which used as a reference control.

Effect of the purified L- asparaginase on the oxidative status

Table 6 shows the effect of L-asparaginase on the level

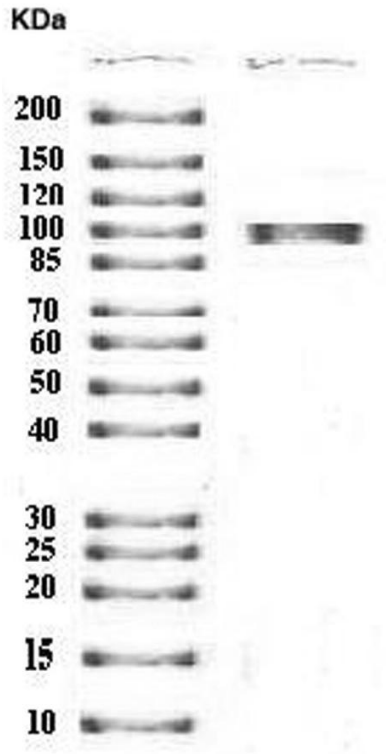

Figure 2. SDS-Polyacrylamide gel electrophoresis of the purified L-asparaginase from *S. halstedii*. 1, Protein marker of molecular weight 200 KDa (Pharmacia fine co., Uppsala, Sweden), 2. Purifed protein after final step of purification.

of MDA as the end product of lipid peroxidation in tumor bearing mice. Results indicate that development of solid tumor is associated with significant elevation of MDA in liver tissue as compared to that of normal control group. Treatment with L-asparaginase resulted in a significant reduction in lipid peroxidation levels. Also, treatment of tumor bearing mice with the purified L-asparaginase leads to remarkable increases in SOD and CAT activities that accompanied reduction in tumor weight, revealing their protective mechanism in tumor prevention (Table 6).

DISCUSSION

The major bioactive compounds obtained from actinomycetes are antibiotics. However, there are varieties of compounds like enzymes that may attract research interest (Basha et al., 2009).

In the present study, *S. halstedii* was selected as potent strain for the production of L-asparaginase. L-Asparaginase formation has shown a firm link to the active cell growth (Savitri and Azmi, 2003; Narayana et al., 2008; Amena et al., 2010).

The *S. halstedii* L- asparaginase precipitated by ammonium sulfate (70%). Many authors (Amena et al., 2010; Kumar and Selvam, 2011) recorded 60% ammonium sulfate saturation and 80%, respectively. The specific activity of L-asparaginase increased from the crude extract to the final Sephadex G-200 purification step, which was approximately 55.2 folds purity. Dhevagi and Poorani (2006) found that L-asparaginase from a marine *Streptomyces* sp. PDK2 has been purified 85-fold with 2.18% recovery in the final Sephadex G-200 purification step.

Dharmaraj and Sumantha (2009), Dharmaraj et al. (2009) and Dharmaraj (2011) found that purified L-asparaginase from *S. gulbargensis*, *S. albidoflavus*, and *S. noursei* MTCC 10469 exhibited molecular weights of 85, 112, and 102 kDa, respectively while *S. halstedii* L-asparaginase contained one protein band with molecular weight of 100 kDa.

The physiological pH is one of the perquisites for anti-tumor activity (Manna et al., 1995; Siddalingeshwara and Lingappa, 2011). Under alkaline pH condition, L-asparaginase becomes a competitive inhibitor (Stecher et al., 1999). This property of the enzyme clarified that the enzyme produced by *S. halstedii* under the present study (optimum pH 8.0) has effective carcinostatic property. The maximum activity in alkaline pH may be due to the balance between L-aspartic acid and L-aspartate. L-Aspartic acid in acid pH has a greater affinity for the active site of the enzyme. In alkaline pH, the balance is shifted toward the aspartate, which is the form with less affinity to the active site enabling, a favorable balance for the connection with the substrate L-asparagine in such alkaline pH (Stecher et al., 1999).

The property of maximum activity of the L-asparaginase at 37°C may be more suitable for complete elimination of asparagine from the body when tumor patient is treated with L-asparaginase (Siddalingeshwara and Lingappa, 2011). Previous data about *P. stutzeri* MB-405, *E. coli*, *Bacillus sp*., pathogenic *E. coli*, and *Aspergillus terreus* KLS optimum temperature for L-asparaginase maximum activity was 37°C (Manna et al., 1995; Li et al., 2007; Aljewari et al., 2010; Moorthy et al., 2010; Siddalingeshwara and Lingappa, 2011) which coincide with *S. halstedii* L-asparaginase optimum temperature for maximum activity in the present study.

The effect of some metal ions and EDTA on *S. halstedii* L-asparaginase coincided to somehow that of Basha et al. (2009) who found that marine actinomycetes isolates S3, S4 and K8 were inhibited by Cu^{2+}, Zn^{2+} and EDTA. The Inhibition of enzyme activity in the presence of Hg^{2+} might be indicative of essential vicinal sulfhydryl groups (SH-group) of the enzyme for productive catalysis (Elshafei et al., 2012). These results indicate that the activity of the enzyme may depend on the presence of sulfhydryl functional groups and the enzyme may not be a metalloenzyme (Basha et al., 2009; Warangkar and Khobragade, 2010).

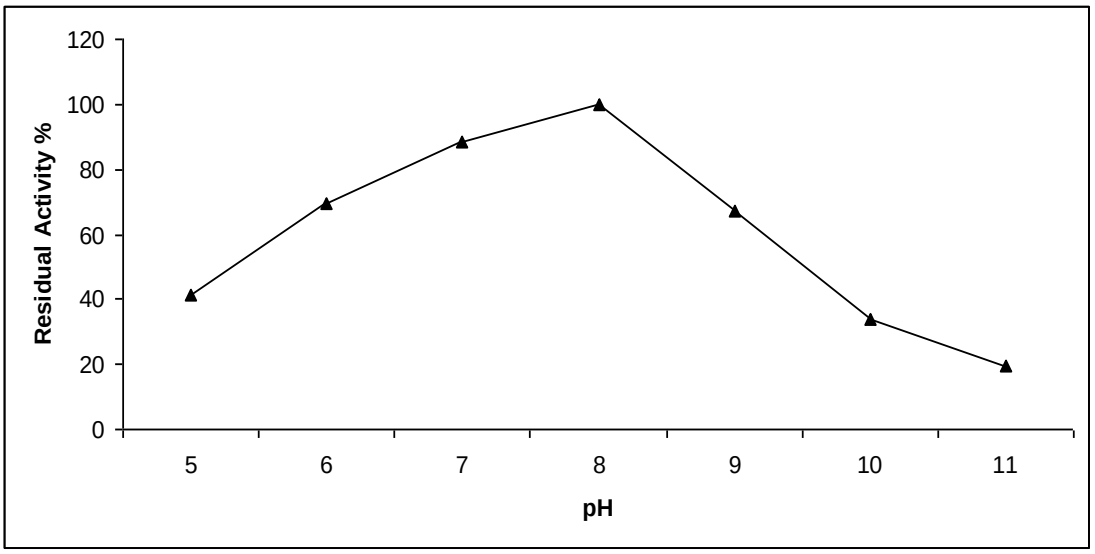

Figure 3. Effect of pH values on the purified L-asparaginase activity from *S. halstedii*.

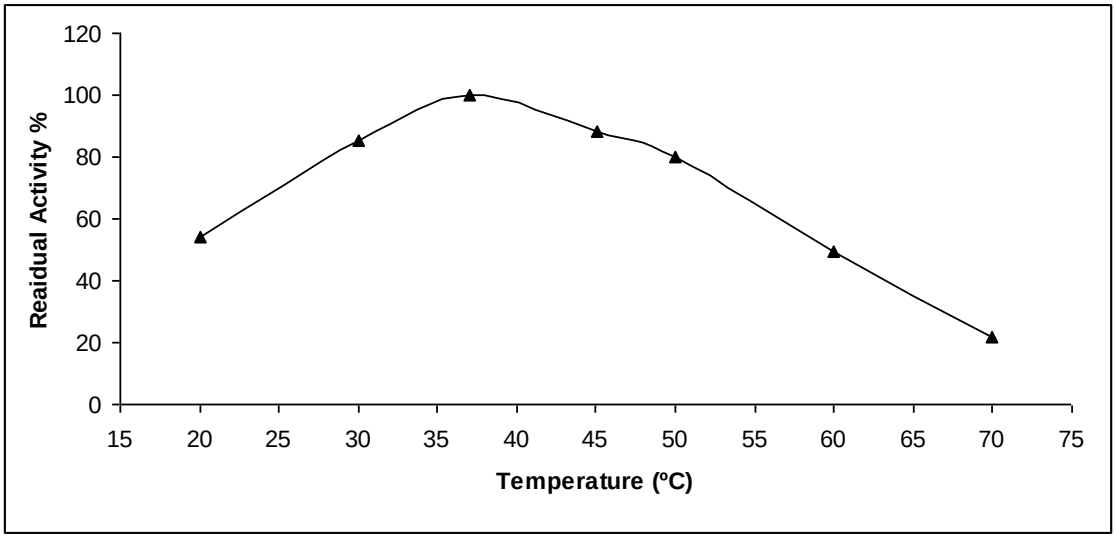

Figure 4. Optimum temperature of the purified L-asparaginase activity from *S. halstedii*.

The K_m and V_{max} of purified L-asparaginase from *S. halstedii* showed high affinity of the enzyme to the substrate (0.1939 mM and 1.22 mM/min, respectively). These kinetic parameters are comparable with those reported for many microorganisms (Basha et al., 2009; Kumar and Selvam 2011).

The purified L-asparaginase from *S. halstedii* exhibits gradual inhibition in the growth of EAC cells *in vitro* as the concentration of the enzyme increased. The inhibition of EAC cells growth coincides with that obtained by many authors (Moharam et al., 2010). Moreover, the enzyme showed a potent inhibitory action on the tumor growth as compared to that of thalidomide anti-tumor drug which was used as a reference control.

Significant results of the antioxidants and lipid peroxidation were obtained after treatment with the purified L-asparaginase, accompanied with reduction in tumor weight, revealing their protective mechanism in tumor prevention. The status of antioxidants and lipid peroxidation were correlated with the pathophysiology of the cancer (Bandebuche and Melinkeri, 2011). This means that, in tumor control, the increased serum MDA levels indicate oxidative stress which may cause DNA damage which is one of the causative factors for cancer

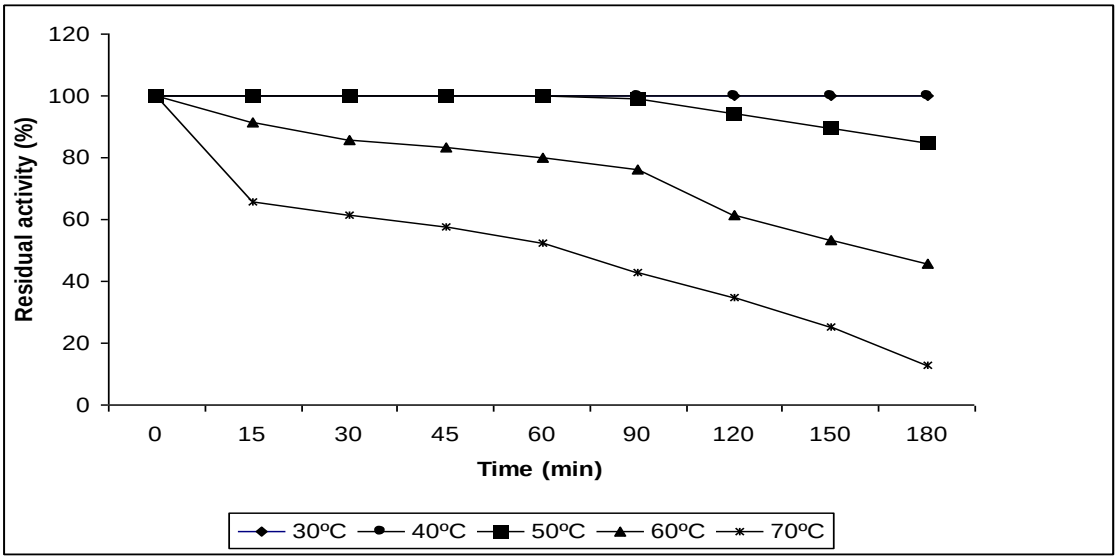

Figure 5. Thermal stability of the purified L- asparaginase from *S. halstedii*.

Table 3. Effect of different concentrations of some metal ions and EDTA on the activity of the purified L-asparaginase from *S. halstedii.*

Concentration (mM)	L-Asparaginase residual activity (%)						
	$KMnO_4$	$CaCl_2$	$FeSO_4$	$ZnCl_2$	$Cu(C_2H_3O_2)_2H_2O$	$Hg(C_2H_3O_2)_2$	EDTA
0.0	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0	100 ±0.0
0.25	92.4 ±0.61	100.0 ±0.0	100.0 ±0.0	98.5 ±0.16	96.8 ±0.22	96.4 ±0.26	64.1 ±0.21
0.5	81.9 ±0.15	100.0 ±0.0	100.0 ±0.0	87.6 ±0.31	87.2 ±0.35	52.9 ±0.27	61.9 ±0.4
1.0	65.6 ±0.17	100.0±0.0	100.0 ±0.0	79.5 ±0.71	72.9 ±0.58	41.3 ±0.14	58.7 ±0.48
5.0	48.0 ±0.11	100.0 ±0.0	100.0 ±0.0	68.2 ±0.21	66.1 ±0.28	33.5 ±0.28	54.7 ±0.17
10.0	36.4 ±0.45	100.0 ±0.0	91.3 ±0.47	56.6 ±0.38	55.0 ±0.11	20.2 ±0.37	52.0 ±0.66

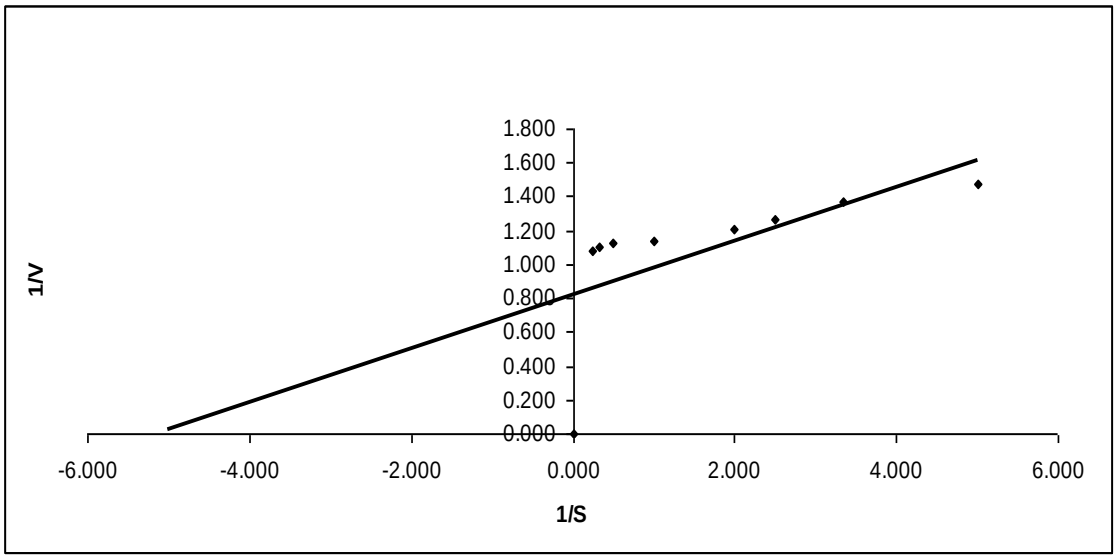

Figure 6. Lineweaver-Burk plot of L-asparaginase from *S. halstedii*. V is the reaction rate and S is the concentration of the substrate.

Table 4. The cytotoxic effect of the purified L-asparginase on EAC cells *in-Vitro.*

Purified L-asparaginase (U/ml)	Cytotoxicity %
10	27.4 ± 6.9
20	50.4 ± 7.3
50	83.4 ± 6.6

Table 5. Effect of the purified L-asparaginase on the growth of solid tumor.

L-asparaginase	Tumor weight (g)	Inhibition%
S. halstedii	0.57 ± 0.13	74.10
Thalidomide	1.31 ± 0.42	40.45
Control	2.20 ± 0.31	-

Table 6. Effect of L-asparaginase and thalidomide on hepatic oxidative status.

Treatment	MDA (nmol /g tissue)	CAT (U/g tissue)	SOD (U/g tissue)
Normal control	69.1 ± 11.0	7.7 ± 1.5	32.6 ± 2.5
Tumor control	197.5 ± 10.8*	3.1 ± 0.5*	19.8 ± 7.0*
L-asparaginase	54.8 ± 4.6$	8.1 ± 0.3$	65.0 ± 6.1$
Thalidomide	188.5 ± 19.0ns	3.5 ± 0.7ns	16.7 ± 3.1ns

*Significant when compared with normal control; $, Significant when compared with tumor control; ns, Non-significant when compared with tumor control.

while low levels of SOD and CAT could be due to the increased utilization of these antioxidants in scavenging the lipid peroxides production which overrides the antioxidant defense leading to increased MDA in serum (Bandebuche and Melinkeri 2011).

In conclusion, this study demonstrates the potential anti-tumor activity of L-asparaginase isolated and purified from strain *S. halstedii* from Egyptian soil. This enzyme exhibited a cytotoxic effect against cancer cell line *in vitro* and in addition, it reduced the growth of solid tumor induced in albino mice. The reduction of tumor size may be attributed to the elevation of CAT and SOD activities as well as the diminishing of MDA.

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Full Length Research Paper

Discovery of antimicrobial activities of a marine diatom *Thalassiosira rotula*

Jian G. Qin[1]*, Trent D'Antignana[1], Wei Zhang[2] and Christopher Franco[2]

[1]School of Biological Sciences, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia.
[2]Flinders Centre for Marine Bioproducts Development, and Department of Medical Biotechnology, School of Medicine, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia.

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This study investigates the antimicrobial activities of a marine diatom, *Thalassiosira rotula* against microorganisms including three Gram-stain positive and six Gram-stain negative bacteria and one species of yeast. Well-bioassays were used to evaluate the ability to inhibit the growth of bacteria with extracts derived from algae using different extraction techniques of cell wall rupture and organic solvents. The extracts of *T. rotula* inhibited the growth of *Vibrio harveyi, Staphylococcus aureus, Micrococcus luteus* and *Bacillus pumilus*. The freeze/thaw algal paste extract without solvent extraction showed the highest inhibition against *S. aureus* and *B. pumilus*. The hexane:tert-butyl-methyl-ether (H:tBME) extract was the only one inhibiting *M. luteus* and the chloroform:methanol (C:M) extract was the only one inhibiting *Vibrio harveyi*. Sonication with beads for 3 min was the most efficient method of releasing the antibiotic substances. In sequential solvent extraction, the vortexed H:tBME extract had a higher ability against *S. aureus, B. pumilus,* and *M. luteus* than the sonicated H:tBME extract or the C:M extract. The 1:1 combination of sonicated H:tBME and vortexed H:tBME extracts did not exceed that of the separate extract against *S. aureus* or *B. pumilus*. However, the combined sequential C:M and vortexed H:tBME extracts had lower ability against *S. aureus* than the vortexed H:tBME extract alone, indicating the presence of antagonistic compounds in the C:M extract. This study indicates that *T. rotula* possess antimicrobial activities but the release of antibiotics depends on physical or chemical rupture of algal cells and extractive solvents.

Key words: Bacteria, algae, antibiotic, organic solvent, sonication, vortex.

INTRODUCTION

Although antibiotics have become an indispensable part of modern medicine, the inappropriate use of antibiotics has resulted in the emergence of antibiotic resistance to bacterial strains (Mukherjee et al., 2005; Fernandez-Delgado and Suarez, 2009; Wright, 2010). Some antibiotic resistant strains pose serious threats to the status quo of public health, which has strengthened the urgency for searching new and effective antibiotics (Gottlieb and Nimmo, 2011; Tello et al., 2012). In primary industries such as aquaculture, bacterial infections contribute to significant losses of aquatic animals and economic income (Desriac et al., 2010; Beardsley et al., 2011; Gao et al., 2012). This situation is exacerbated by the increasing occurrence of bacteria that are resistant to antibiotics in cultured and wild fish (Kitto et al., 1999). With an escalating number of species for aquaculture and the ever-increasing need to achieve the highest possible productivity in a given system, the incidence of bacterial

*Corresponding author. E-mail: jian.qin@flinders.edu.au. Tel: +61 8 8201 3045. Fax: +61 8 8201 3015.

infection among cultured fish has become increasingly common. Successful culturing of many species will therefore require the development of new antibiotics in an attempt to control pathogens such as *Vibrio harveyi*, *Vibrio vulnificus*, *Streptococcus* sp., and those that have become resistant to common antibacterial agents (Wang, 1999). Clearly, it is imperative to discover new antibiotics, not only to combat resistant strains, but to reduce the possibility of further increasing the antibiotic resistance of the bacterial community.

The marine environment is viewed as a new and underexplored source of potentially useful bioactive compounds (Davidson, 1995), particularly given that the microbial, plant and invertebrate diversity in the marine environment far exceeds that of terrestrial habitats (Carte, 1993). The potential for using microalgae to yield novel compounds is strengthened by the fact that microalgae are the foundation of the food chain to support the most of aquatic biomass (Conti and Scardi, 2010). Cconsequently, microalgae may be the primary source for some of the novel molecules found in marine invertebrates.

Microalgae represent a large and underexplored resource of antimicrobial compounds (Guedes et al., 2011). Furthermore, recent advances in algal culture techniques have placed microalgae in a unique position over many other marine organisms, as algae can be cultured under conditions that maximise the production of the desired compound (Chisti, 2007). Thus, the investigation of marine microalgae for their antimicrobial activity is likely to find compounds that can be used in the aquaculture industry. Microalgae are known to produce compounds intracellularly and extracellularly. However, as a large proportion of these compounds is not excreted but remains within the cells (Guedes et al., 2011; Das and Pradhan, 2010), solvent extraction is widely used to extract these secondary metabolites in algae (Zhao et al., 2004). Algal antimicrobials are structurally diverse and it is common for an alga to produce a variety of antibiotic compounds with different spectra of antibiotic activity (Metting and Pyne, 1986). Clearly, one solvent system would most likely be inadequate to extract all chemical classes of antimicrobials produced by an algal species. For instance, Lustigman (1988) extracted the biomass of *Dunaliella salina* in a range of solvents and found that the solvent of butanol extract produced the largest zones and the broadest spectrum of activity, whereas the methanol and chloroform extracts showed no activity. In contrast, Chang (1993) used a variety of solvents to extract *Dunaliella primolecta* and found methanol extracts to be the most active while hexane extracts were inactive. Alternatively, Kellem et al. (1989) examined the antifungal activity of a number of microalgae and found that in some species the methanol extracts were active whereas in others the hexane extracts that were active.

Therefore, different solvents need to be used to determine the true antibiotic potential of an algal species, as the size and polarity of algal metabolites will ultimately determine the metabolites and their solubility in an organic solvent. To recognise the antibiotic potential of an algal species, extracts need to be tested against a broad spectrum of bacteria and fungi. For example, Cooper et al. (1983) reported that aqueous extracts of *Skeletonema constatum* exhibited a broader spectrum of activity when compared to the organic extracts. This implies that there are multiple antibiotics present with different polarities and spectra of activity. Obviously, the broader activity of the aqueous extracts would not be detected if a narrow spectrum of bacteria would be used to test the activity of the extracts. Therefore, it is necessary to extract the algal biomass using a number of solvents which represent a range of polarities (non-polar to polar solvents). Similarly, a suite of test microorganisms should be used if the true antibiotic potential of an algal species is to be recognised.

Various techniques have been used to liberate metabolites from their cells and increase the efficiency of solvent extraction, such as changing ambient salinity to exert osmotic shock before solvent extraction (Viso et al., 1987; Lustigman, 1988), mixing the solvent with algal biomass (Kellam and Walker, 1989), homogenising algal materials (Miura et al., 1993; Wang, 1999) and sonicating algal biomass (Chang et al., 1993; Ohta et al., 1995). Although freeze/thaw extraction is not extensively used in algal extractions, it is a common method to extract bioactive materials that are thermally unstable (Silvia et al., 1998). As raw materials with a small particle size are easy for compound extraction (Silvia et al. 1998), the procedure of cell rupture should be carefully chosen before solvent extraction. Algal cells are diverse in shape, size, cell wall thickness and cell wall composition (Richmond, 2004). Such morphological characteristics may influence the efficiency of a particular extraction technique. Thus, methods of physical rupture to assist penetration of a solvent into the cellular material need to be refined for an algal species of interest if one is to maximise antibiotic yield from the algal biomass.

The aims of this study were to (1) examine the *in vitro* antimicrobial activity of *Thalassiosira rotula* extracts, an algal species commonly used in aquaculture hatchery, against a suite of microorganisms using well bioassays, (2) identify the type of algal extracts with the highest activity, (3) explore extraction techniques in an attempt to increase the antibiotic yield, and (4) identify an appropriate solvent system that would maximise the yield of a particular antimicrobial.

MATERIALS AND METHODS

Experimental organisms

The diatom *T. rotula* was obtained from the Australian National Algae Culture Collection in the Commonwealth Scientific and Industrial Research Organisation (http://www.csiro.au/Organisation-

Table 1. Microorganisms used to test the antimicrobial activity of algal extracts.

Microorganism	Source
Gram-stain positive	
Staphylococcus aureus (Sa)	FMC[1]
Bacillus pumilus (Bp)	FMC
Micrococcus luteus (Ml)	FMC
Gram-stain negative	
Escherichia coli (Ec)	FMC
Enterococcus cloacae (Ecl)	FMC
Gram-stain negative (marine)	
Vibrio natrigens (Vn)	FU[2]
Vibrio harveyi (Vh)	FU
Vibrio fischerii (Vf)	FU
Yeast	
Candida albicans (Ca)	FMC

[1]FMC: Flinders Medical Centre; [2]FU: Flinders University, School of Biological Sciences.

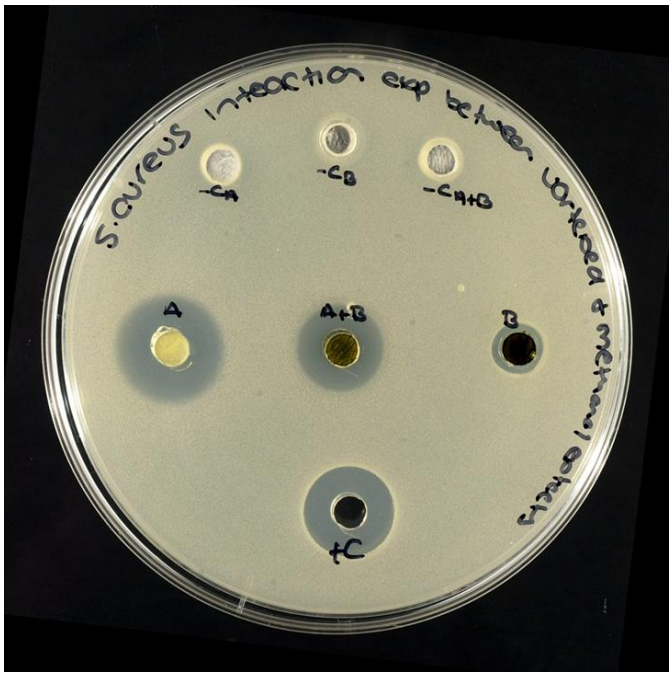

Figure 1. Illustration of well assay to test *T. rotula* extract inhibition on *S. aureus*. The transparent areas are the inhibition zones by -CA (hexane solvent control), -CB (chloroform/methanol control), -CAB (hexane + chloroform/methanol control), A (hexane extract), B (chloroform/methanol extract), AB (hexane + chloroform/ methanol extract), and +C (vancomycin control).

Structure/National-Facilities/Australian-National-Algae-Culture-Collection/Microalgae-Supply-Service.aspx), Tasmania, Australia, to examine its antimicrobial activity. This alga was grown in natural seawater enriched with F2 media. Algal stocks (100 ml) were maintained and refreshed every two weeks by transferring 10 ml of stock culture into 90 ml of media. Stock cultures of high cell density were used to seed 1 L Schott-bottles filled with 900 ml of media. One-litre cultures were maintained under continuous light at 19°C and aerated at a rate of 100 ml/min. The 1 L cultures were inoculated in 20 L carboys. Algal cultures were subjected to a photoperiod of 12 h light:12 h dark and illuminated by a bank of fluorescent tubes at 2000 lux. Cultures were maintained at 23 ± 1°C and were continuously injected with air containing 5% (v/v) CO_2 at a flow rate of ~600 ml/min.

Nine microorganisms were used to evaluate the antimicrobial activity of the algal extracts (Table 1). These microbes were cryogenically stored at -80°C in 20% glycerol prior to the study. To obtain single colonies, glycerol stocks of *Vibrio* spp. were streaked on to the Hasting agar (Hasting, 1986), and the other micro-organisms were streaked onto the nutrient agar. Agar plates were then incubated at 25°C. After 24 h incubation, plates were removed from the incubator and stored in a fridge at 4°C. These plates were replaced with freshly streaked plates every two weeks.

Testing antimicrobial activity of algal extracts

Using a Sorvall GSA fixed angle rotor in a Sorvall RC-5B refrigerated centrifuge (DuPont Instruments), 1.5 L algal culture was centrifuged (5000 *g*) at 0°C for 10 min. The biomass pellets was then resuspended in the remaining supernatant and transferred to 50 ml centrifuge tubes. This concentrated algal culture was then centrifuged at twice 4500 *g* for 2.5 min. The biomass pellet was used for extraction.

The well bioassay was used to examine the antimicrobial activity of microalgal extracts (Figure 1). Aliquots (10 ml) of the Tryptone soya broth or hasting broth for *Vibrios* were inoculated with a single colony and allowed to grow overnight at 25°C. The optical density (OD) of each overnight culture was measured at 600 nm using a Shimadzu (UV -160 A) spectrophotometer, and adjusted to an OD of 0.1 by the addition of sterile, saline water (0.9% NaCl in water, w/v). Diluted overnight cultures were used to bulk seed the bioassay agar or hasting agar at 2% (v/v) on the agar. Seeded agar was poured on to plates to a depth of 5 mm and once the agar had set, 5 mm wells were punched in to the bioassay plate. To each well, 40 µl of a given solvent extract or solvent control was then added. Plates were then incubated overnight at 25°C for each microorganism. After 18 h, zones of inhibition were measured to the nearest 0.5 mm using a pair of vernier callipers.

Extraction protocols for antibiotic compounds

Upon reaching the stationary phase, 15 L of the well-mixed algal culture was harvested, and divided into ten 1.5 L culture samples and subsequently centrifuged. The fresh weight of each of the 10 algal pellets was then recorded. The volume of one algal pellet was adjusted to 1.5 ml by adding distilled water, and then stored at -18°C. In addition, a 10 ml sample of the supernatant from each 20 L culture was stored at -18°C to test for antimicrobial activity.

The antimicrobial ability of algae was tested on (1) algal free culture media, (2) freeze/thaw algal paste (freeze for 24 h then thaw at room temperature), (3) water as a solvent, (4) hexane:tert-butyl-methyl-ether (H:tBME, 1:1 v/v) as a solvent, and (5) chloroform:methanol (2:1, v/v) as a solvent (Table 2). One solvent was added to algal pellets in triplicate with each pellet receiving 1 ml solvent. To improve the effectiveness of extraction, three physical treatments were used:sonication only, sonication with 1 g

Table 2. Inhibition zone diameters (mm) produced by *Thalassiosira rotula* crude extracts, against nine microorganisms. Zones of inhibition are measured as the means ± SD of the diameter of the zones (well diameter = 5 mm). Different letters in the same column represent significant differences ($P < 0.05$). Abbreviations of test microorganisms refer to Table 1.

Extract	Test microorganism								
	Marine					Terrestrial			
	Vn	Vh	Vf	Ec	Ecl	Sa	Bp	Ml	Ca
Algal free culture media	0	0	0	0	0	0	0	0	0
Freeze/thaw paste of algal biomass	0	0	0	0	0	14.2 ± 0.3^{c}	14.5 ± 0.5^{c}	0	0
Water	0	0	0	0	0	9.7 ± 0.6^{b}	7.8 ± 0.3^{b}	0	0
Hexane: tert-Butyl-Methyl-Ether (H:tBME)	0	0	0	0	0	9.5 ± 0.1^{b}	13.8 ± 0.3^{c}	12.5 ± 0.5	0
Chloroform:methanol (C:M, 2:1)	0	7.8 ± 0.3^{b}	0	0	0	7.3 ± 0.6^{b}	7.5 ± 0.5^{b}	0	0
C:M solvent control	0	5.8 ± 0.3^{a}	0	0	0	6.0 ± 0.1^{a}	6.0 ± 0.1^{a}	0	0
H:tBME solvent control	0	0	0	0	0	0	0	0	0

glass beads (212 to 300 μm diameter); and vortexing. Using the well bioassay, the algal extracts obtained from the different extraction methods were tested for antimicrobial activity against each microorganism (Table 1). Each extract was tested on three separate bioassay plates. Each solvent was also used as a control to measure any activity due to the solvent.

Sonication time

The effect of sonication time was investigated to identify whether sonication was degrading the antibiotic material. Upon reaching the stationary phase, 13.5 L of well-mixed *T. rotula* culture was harvested and divided into nine 1.5 L aliquots. The 1.5 L culture samples were centrifuged and the wet weight of each algal pellet recorded. A volume of 1 ml of H:tBME was then added to each pellet along with 800 mg of glass beads.

The nine extraction mixtures were divided into three groups with three replicates. Each group was subjected to sonication for 1, 3 and 6 min (100 μA, Qsonica model Q55) on ice, respectively. Once sonicated, the total solvent (H:tBME) volume in each sonicated mixture was brought to 5 ml and allowed to stand for 1 h. Each extraction mixture was then centrifuged for 2.5 min at 4500 *g.* The organic layer from each sample was removed and placed into 10 ml tubes. Extracts were then concentrated under a stream of nitrogen gas to yield 500 μl extracts and stored in eppendorf tubes at -18°C. Extract activities were then examined against *Staphylococcus aureus* and *Bacillus pumilus* using the well bioassay. Each extract was tested once on three separate bioassay plates.

Sequential extractions using different solvents

The screening study suggested that a number of antimicrobials might exist in *T. rotula*. To identify the antimicrobials produced and their solvent affinities, sequential extractions of the algal biomass were performed. Upon reaching the stationary growth phase, 4.5 L of *T. rotula* was harvested and divided into three 1.5 L samples for centrifuging to yield three algal pellets for sequential extraction outlined below.

Vortexed H:tBMe extraction

Algal pellets of *T. rotula* were initially extracted in 5 ml H:tBME, vortexed for 5 min and allowed to stand at room temperature for 1 h. Subsequently, each extraction mixture was centrifuged for 2.5 min at 4500 *g*. The organic extracts from each extracted pellet were then transferred to separate 10 ml centrifuge tubes. Following the removal of the organic extract, each extracted pellet was washed with a further 5 ml H:tBME, vortexed for a total of 4 min and then left to stand for 20 min. Each mixture was centrifuged as above and the organic supernatants transferred to separate, labelled 10 ml tubes.

Sonicated H:tBME extraction

Following the vortexed H:tBME extraction, a volume of 1ml of H:tBME was added to each of the three extracted pellets along with 800 mg of glass beads. Each was then sonicated on ice for 3 min. Once sonicated, the total solvent volume in each mixture was brought to 5 ml and left to stand for 1 h. Subsequently, each mixture was centrifuged and the organic supernatants were placed in individual 10 ml tubes. The sonicated algal material was then washed following the washing procedure implemented in the vortexed H:tBME extraction. Each wash was kept in separate 10 ml tubes.

Chloroform:methanol (C:M) extract

After removal of the organic layer, each algal pellet was dried under nitrogen gas to remove any remaining H:tBME. Once free of the previous solvent system, each pellet was extracted in 5 ml of C:M solvent, vortexed for 5 min, and left to stand for 1 h. The organic supernatant was kept in separate 10 ml tubes. Each extracted pellet was then washed with 5 ml of C:M (2:1 v/v) solvent following the washing procedure implemented in both H:tBME extractions. Using a stream of nitrogen gas, algal pellets were dried of any residual C:M solvents. Once dry the remaining algal material in each extraction tube was extracted and then washed with distilled water. The procedure of extracting and washing was identical to that preformed for the sequential C:M solvent extraction. Sequential extracts were subsequently examined for antimicrobial activity against *B. pumilus*, *S. aureus* and *M. luteus* using the well bioassays. In addition, solvent controls (chloroform:methanol, H:tBME and water) were included on each bioassay plate.

Antibiotic activities of combined extract from different solvents

To determine the presence of antagonistic compounds, we

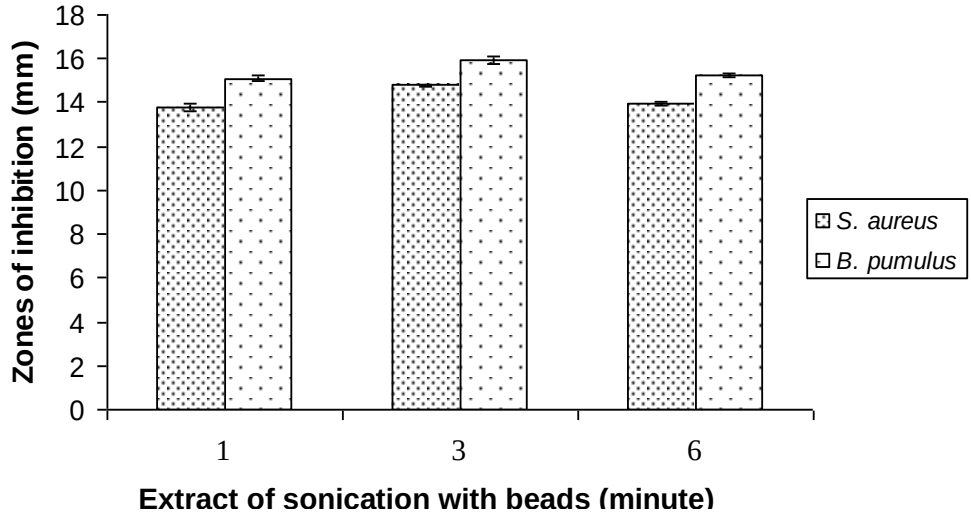

Figure 2. Effect of sonication time on *T. rotula* H:tBME extract activity. Activity in the well bioassay was measured as mean zones (±SE) of inhibition in triplicate for *T. rotula* H:tBME extracts against *S. aureus* and *B. pumilus*. Extract biomass was subjected to sonication of 1, 3 or 6 min.

combined several of the sequential extracts to examine if their combined activity differed from their individual activity. The vortexed H:tBME extract and the sonicated H:tBME extract were each diluted to 50% of their original concentration by the addition of H:tBME (1:1). A third extract was then formed by mixing the vortexed H:tBME extract with of the sonicated H:tBME extract (1:1). These extracts were then bioassayed in triplicate against *B. pumilus* and *S. aureus*. The methodology outlined above was then repeated using the vortexed H:tBME extract and the sequential C:M extract. Extracts and their respective solvent control were bioassayed against *S. aureus*.

Statistical analysis

Single factor analysis of variance was conducted using statistical software (SPSS, 18.0) to determine whether extract methods were significantly different from that of the solvent control. All zones of inhibition were converted to logarithmic values before being analysed to improve data normal distribution. Multiple comparisons were then preformed using the Bonferroni post hoc test when the main effect of treatments was significant. Linear regression was performed to determine the relationship between extract concentrations and the zone size of inhibition. Differences between the means were considered significant at the level of $P < 0.05$.

RESULTS

Antimicrobial activities of algal extracts

Antimicrobial activity was evaluated by measuring the diameter of the zone of inhibition in the well bioassay. The algal cell free culture media did not inhibit the growth of any microorganisms, but the freeze/thaw paste, water, H:tBME and C:M extracts were active against *S. aureus* and *B. pumilus* (Table 2). The freeze/thaw paste extract exhibited the highest antimicrobial activity of any algal extract, inhibiting *S. aureus* and *B. pumilus* to a similar degree. The activity of the H:tBME extract against *B. pumilus* was similar to that of the freeze/thaw paste. However, the activity of the H:tBME extract against *S. aureus* was significantly lower ($P = 0.001$) than that of the freeze/thaw paste extract. Comparing all extracts, the H:tBME extract exhibited the highest activity against *M. luteus* and the C:M extract was active against *V. harveyi* ($P = 0.001$) when compared to the C:M solvent control.

The cell free supernatant was inactive against any microorganisms, while the freeze/thaw paste and the H:tBME extract were consistently the most active extracts against *B. pumilus*. The C:M and water extracts were the only extracts active against *V. harveyi*. The activity of the freeze/thaw paste was usually higher than that of the best solvent extract. *S. aureus* and *B. pumilus* were the most sensitive microbes, while *Escherichia coli*, *Escherichia cloacea* and the yeast *C. albicans* were not inhibited by any of the algal extracts. The C:M solvent extract could inhibit the growth of *V. harveyi*, *S. aureus* and *B. pumilus*compared to the C:M control ($P<0.001$).

Effects of cell rupture methods on antimicrobial activities

Sonicating for 1, 3 or 6 min had some effect on the activity of the H:tBME extracts in the well assay. Multiple comparisons indicated that the 3 min sonication treatment had significantly higher activity against *B. pumilus* than both the 1 min and 6 min sonication treatments ($P = 0.002$ and $P = 0.008$, respectively, Figure 2). Extracts sonicated for 3 min were more active against

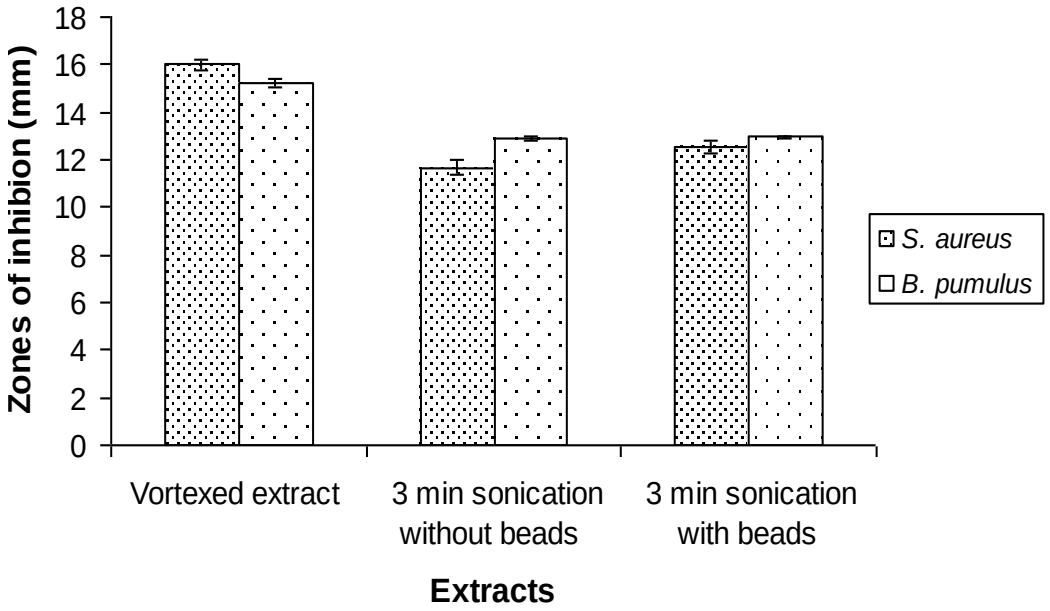

Figure 3. Effect of vortexing and sonication with or without beads on the activity of *T. rotula* H:tBME extracts. Activity in the well bioassay was measured as mean zones (±SE) of inhibition in triplicate for *T. rotula* H:tBME extracts against *S. aureus* and *B. pumilus*. Treatments included vortexing the biomass in H:tBME, and sonicating the algal biomass for 3 min with and without beads before extracting in H:tBME.

S. aureus than those sonicated for 1 min (P = 0.022), although the 3 min sonication appeared slightly more active against *S. aureus* than the 6 min sonication treatment (P = 0.112).

Since the 3 min sonication treatment showed best extraction results, the 3 min sonication treatment was used as a reference to compare the activity of vortexed extracts and sonicated extracts with and without glass beads. This treatment was chosen as it previously displayed the highest activity, and would act as a control for other extraction methods.

Sonicating *T. rotula* biomass with or without beads did not significantly alter the H:tBME extract's ability to inhibit the *B. pumilus* (P = 0.99), but the use of beads in sonicating did significantly improve the extracts ability to inhibit *S. aureus* (P = 0.023, Figure 3). Of all the extraction treatments using the well bioassay, the vortexed H:TBME extraction treatment exhibited the highest antimicrobial activity against both *S. aureus* and *B. pumilus* (P < 0.05, Figure 3).

To ensure the observed zones of inhibition increased as a function of extract concentration, the *T. rotula* extracts of increasing concentration were bioassayed against the most sensitive microbe *B. pumilus*. Linear regression was then performed to determine the relationship between extract concentration and zones of inhibition (Figure 4). Based on the trend, the activity of *T. rotula* extracts showed an increase with extract concentrations (R^2 = 0.95, P < 0.001).

Activities of single sequential solvent extracts

Multiple solvent systems detected the presence of more than one antimicrobial substance. To gain a perspective into the number of antimicrobials produced by *T. rotula* and identify the most appropriate solvents to extract them, sequential extractions of the biomass were performed (Table 3).

The vortexed H:tBME extract for *T. rotula* exhibited the highest activity against *S. aureus*, *B. pumilus* and *M. luteus* (P < 0.05, Table 3). Sequential C:M extracts showed activity against all microbes. Its activities against these microorganisms were lower than those of the vortexed H:tBME extractions, but similar to those of the sonicated H:tBME extract. The extraction controls of the water and H:tBME did not show any antimicrobial activity, but the C:M solvent inhibited *B. pumilus*.

Activities of combined sequential extracts

The sonicated H:tBME extract was combined with the vortexed H:tBME extract (v:v=1:1). The combined extract activity was then compared to the activity of the individual extracts which had been diluted by 50% (Table 4).

The vortexed H:tBME (½ conc) extract and the combined extract had a higher activity than the sonicated H:tBME (½ conc) extract (P< 0.05). When tested against *B. pumilus*, the combined extract exhibited the same

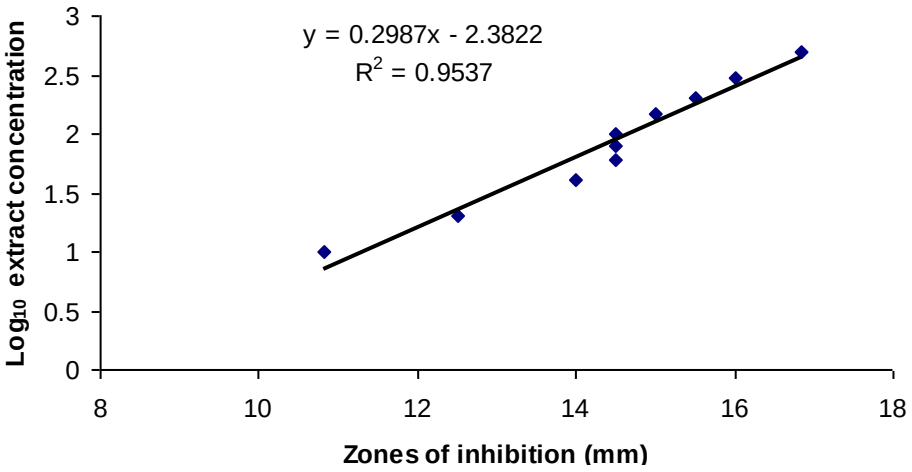

Figure 4. Relationship between extract concentration and zones of inhibition produced by *T. rotula* H:tBME extracts against *B. pumilus*.

Table 3. Antimicrobial activity in sequential solvent extracts of *Thalssiosira rotula* as exhibited in well bioassays. Zones of inhibition include well diameter (5 mm).

Extracting order	Extract	Microorganism[a]		
		Bp	Sa	Ml
1	Vortexed H:tBME extract	17.4 ± 0.5[c]	18.8 ± 0.3[c]	14.7 ± 0.1[c]
2	Sonicated H:tBME extract	9.6 ± 0.1[b]	7.5 ± 0.5[b]	9.1 ± 0.5[b]
3	C:M extract	7.7 ± 0.3[b]	7.3 ± 0.3[b]	7.7 ± 0.5[b]
	C:M solvent control	6.0 ± 0.1[a]	0	0
	Water extract control	0	0	0
	H:tBME control	0	0	0

Different letters in the same column represent significant differences (P< 0.05). Abbreviations of the microorganisms and solvent refer to Table 1.

Table 4. Mean zones of inhibition produced against *Staphylococcus aureus* and *Bacillus pumilus* when the *T. rotula* vortexed H:tBME extract was combined with the sonicated H:tBME extract. Activity was compared to the zones of inhibition (mm) produced by the individual extracts.

Extract	*S. aureus*	*B. pumilus*
Vortexed H:tBME extract	16.5 ± 0.1[b]	15.5 ± 0.1[b]
Sonicated H:tBME extract	6.3 ± 0.3[a]	7.5 ± 0.1[a]
Combined H:tBME extract	15.7 ± 0.3[b]	15.5 ± 0.1[b]
H:tBME solvent control	0	0

Different letters in the same column represent significant differences (P < 0.05).

degree of activity as that of the vortexed H:tBME (½ conc) extract alone. Against *S. aureus*, zones of inhibition appeared to be smaller in the combined extract when compared to the vortexed H:tBME (½ conc) extract. This difference, however, was not significant (P = 0.206).

Similarly, the vortexed H:tBME extract was combined with the sequential C:M extract (v:v=1:1),and the antimicrobial activities are reported in Table 5. The vortexed H:tBME (½ conc) extract and the combined extract were significantly more active than the sequential C:M (½ conc) extract as depicted by their zones of inhibition against *S. aureus*. In addition, the results clearly showed

Table 5. Mean zones of inhibition produced against *Staphylococcus aureus* when the *T. rotula* vortexed H:tBME extract was combined with the sequential C:M extract. Activity was compared to the zones of inhibition (mm) produced by the individual extracts. Different letters in the same column represent significant differences ($P < 0.05$).

Extract	*S. aureus*
Combined extracts of vortexed H:tBME and sequential C:M	13.5 ± 0.5^{b}
Vortexed H:tBME extract	17.5 ± 0.1^{c}
Sequential C:M extract	8.0 ± 0.5^{a}

that the vortexed H:tBME extract had a signi-ficantly reduced activity against *S. aureus,* when com-bined with the C:M extract ($P = 0.001$).

DISCUSSION

Screening microalgae for antimicrobial activity

This study demonstrates that *T. rotula* had the highest antimicrobial activity against *S. aureus, B. pumilus,* and *M. luteus* and was active against *V. harveyi*, but had no antibiotic activity on *V. natrigens, V. fischerii, E. coli, E. cloacae* and a yeast *C. albicans.* A variety of solvents for the extraction of algal biomass have been reported with hexane, methanol, chloroform, benzene and iso-propanol being the most frequently used (Fastner et al., 1998; Herrero et al., 2005; Kang and Sim, 2007). In comparison, this study shows that the most non-polar solvent system (that is, H:tBME) consistently extracted the compounds with the highest activity against Gram-positive microbes. Thus, the antimicrobial compounds active against *Vibrio* appear to have a higher polarity than those active against the Gram-positive microbes since the *Vibrio* was only inhibited in the polar solvent of chloroform and methanol.

The presence of antibacterial activity in natural sea-water is well known (Taskin et al., 2007; Ibtissam et al., 2009; Mandeel et al., 2010; Salem et al., 2011). Lustigman (1988) found that in addition to the algal biomass having activity, the culture filtrate of *Dunaliella salina* was also active against Gram positive, Gram negative and marine bacteria. In addition, Trick et al. (1984) also reported that the antibiotic β-diketone was released into the culture filtrate by *Prorocentrum minimum*. However, similar to Kellem and Walker (1989), this study observed no antimicrobial activity in the culture supernatant. This may have been because the culture filtrate was too dilute, or because of physiological, environmental and experimental conditions.

The current study has demonstrated for the first time, the strong antibacterial activity in the marine diatom *T. rotula*. In the past, a *Thalassiosira* sp. was examined by Visoet al. (1987) and found to be active against *S. aureus* and *M. luteus*, but inactive against *E. coli* and *C. albicans*. Furthermore, *T. nana* and *T. decipiens* were reported to be active against a number of soil bacteria (Kitto et al., 1999). Thus, it appears that the genus *Thalassiosira* has the ability to produce antimicrobial secondary metabolites.

Antimicrobial activity is evaluated on the ability of an algal extract to inhibit certain microbes. It is therefore conceivable that under a different set of test microbes, the activity of the algal extract could have been signify-cantly different. For instance, Ohta et al. (1994) investigated the activity of *Chlorococcum* HS-101 against seven strains of methicillin resistant *S. aureus* and found the zone sizes of inhibition ranged from 17.7 ± 1.5 to 28.3 ± 1.7 mm. Viso et al. (1987) found that extracts con-taining eicosapentaenic acids from the diatom *Asterionella japonica* exhibited strong antibiotic activity after a short period of illumination. Similarly, Miura et al. (1993) observed that extracts of *Chlorella* were inactive against *B. subtilis* in the dark, but became active when illuminated. Thus, it is possible that the alga in the current study may have produced light sensitive antimicrobials, but their production would not have been recognised because the bioassay plates were incubated in dark.

Effects of cell rupture methods on antimicrobial substance release

Numerous techniques have been used to release antimicrobial compounds into the extracting solvents (Vlachos et al., 1996; Herrero et al., 2005; Shanmughapriya et al., 2008). This study found that sonicating with glass beads (240 to 300 μm) was the most efficient way to release the antimicrobial com-pounds from the biomass of *T. rotula*. However, in the well bioassay the vortexed extract exhibited an activity significantly higher than that of the sonicated extracts, suggesting that the method of physical rupture of algal cells may interfere with the effectiveness of antibiotic property in algae.

Zones of inhibition were reduced when algal pellets were sonicated for 6 min. This indicates that sonicating beyond 3 min may degrade some of the antibiotic mate-rial. Sonication can generate very high local temperature

around the probe, which could degrade the antibiotic material if it would be heat sensitive. This scenario is possible considering that antibiotics produced by *D. primolecta* are degraded when temperatures exceed 40°C (Chang et al., 1993) and that the antibiotic material of *D. salina* is degraded at temperatures exceeding 25°C (Lustigman, 1988). Thus, it is possible that the decrease in antibiotic activity may result from degradation of antibiotic material due to overheating in the 6 min sonication procedure.

This may partially explain why the algal paste in the screening experiment had an activity higher than that of the H:tBME solvent extraction. These antibiotic compounds seem to be relatively polar, and would therefore have a low affinity for the H:tBME which is a relatively nonpolar solvent system. Thus, to extract out more of these polar compounds, a more polar solvent system should be used and the extraction time should be increased. Although sonication could effectively break cell walls, it may degrade the antibiotic activity of the extract. Therefore, increasing the extraction time in a particular solvent system should be considered during extraction.

Sequential solvent extraction of algal biomass

It is commonly observed that a microalgal species produces multiple antibiotic substances, each with different spectra of activity (Miura et al., 1993; Ohta et al., 1995). *T. rotula* was no exception, exhibiting the ability to produce a diverse array of antimicrobial compounds, that is, some are only active against *S. aureus*, while others display broader spectra of antibacterial activity. In the sequential solvent extraction, the H:tBME solvent extracted much more antibiotic compounds than the C:M solvent extracts from the *B. pumilus*, *S. aureus* and *M. luteus*, suggesting the use of C:M solvent is not appropriate.

In the sequential extraction with physical rupture on algal cells, the vortexed H:tBME extract showed similar ability of inhibition against *S. aureus* and *B. pumilus* as the combined extracts of vortexed and sonicated H:tBME solvent, suggesting that the one step of vortexed extraction is adequate and time efficient.

It is well known in the clinical application of antibiotics that the presence of multiple antibiotics together commonly results in decreased activity (Krogstad and J, 1986). In this study, the combined extracts of vortexed H:tBME and sequential C:M reduced the inhibitory ability against *S. aureus*compared to the vortexed H:tBME extract alone, suggesting the presence of antagonistic compounds in the C:M extract. This study did not identify the property of antibiotic compounds and the mechanism of compounds. However, future research is required to determine the identity of the antagonistic interactions between antibiotics.

Conclusion

This study reports the presence of antibacterial compounds in *T. rotula* which inhibit the growth of *V. harveyi, S. aureus, Bacillus pumilus* and *M. luteus*. Vortexed H:tBME was the most effective extract method, but the use of glass beads for algal cell rupture could aid the release of antibiotic substances. However, the time of sonication may need to be adjusted for an individual species of interest. In a screening situation, 1 to 3 min sonication with beads would appear to be sufficient to release the majority of the antibiotic material from the biomass, particularly when performing sequential extractions. The presence of antagonists in the extracts of *T. rotula* suggests that the use of a crude algal extract without separation may underestimate the true antibiotic potential.

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Full Length Research Paper

Slime producing, heavy metals and antibiotics resistance in *Aeromonas hydrophila* isolated in Tunisia

Nourhene Saidi*, Rihab Lagha, Fethi Ben Abdallah, Karima Bekir Rokbani and Amina Bakhrouf

Laboratory of Analysis, Treatment, and Valorization of Environment and Products Pollutants, Department of Microbiology, Faculty of Pharmacy, Street Avicenne, Monastir, Tunisia.

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Aeromonas hydrophila **strains isolated from different naturally polluted environments (ten from wastewater, six from bay used for aquaculture, eight from sea coast water and six from fish) were subjected to 13 antibiotics, and to four heavy metals (Copper, Cobalt, Zinc and Mercury) by using agar diffusion and agar dilution methods, respectively. In addition, effect of heavy metals on slime production was also investigated. Results of the antibiotic resistance agreed with those of heavy metals resistance, however, treated wastewater and bay strains were much tolerant than seawater and fish bacteria. The range of metal concentrations that was tolerated in the liquid media yielded information on the tolerance levels of *A. hydrophila* to different tested concentrations of metals. Copper and zinc were the best tolerated metals. Mercury was the most toxic component for all bacteria. Almost all *A. hydrophila* produced slime and a small number of strains have changed their morphotype under the heavy metals concentration. Our results have shown that Tunisian aquatic biotopes have a significant proportion of antibiotic and heavy metal resistant to *A. hydrophila.***

Key words: Antibiotic resistance, *Aeromonas hydrophila*, heavy metals and slime producing.

INTRODUCTION

The anthropogenic contamination of the environment with heavy metals is a serious problem. Aquaculture (Burridge et al., 2010) and agricultural practices (Han et al., 2002; Nicholson et al., 2003) contribute to this world wide pollution due to diverse applications of metals in feed additives, organic and inorganic fertilizers, pesticides, and anti-fouling products. Fish farmers frequently use pharmaceuticals (such as antibiotics) and metal containing products to prevent fouling, to feed and to treat fish in order to limit the spread of infections (Burridge et al., 2010).

Therefore, bacterial communities of aquacultures are strongly exposed to the combination of heavy metals and antibiotics. The exposure to both antimicrobial substances may increase the likelihood of selection and co-selection of antibiotic resistance. Moreover, the high nutritional value and the relatively low cost of wastewater, excreta, and sewage sludge convert such heavy metal containing waste to valuable fish feed, especially in developing countries (WHO, 2006).

In Tunisia, the persistence and proliferation of antibiotics and heavy metals resistance in bacterial pathogens, belonging to the *Aeromonas hydrophila*, in aquatic environments represents a considerable public health concern. Subsequent measures to control the emergence and propagation of antibiotic resistance have encountered limited success, and it persists in spite of the restricted use of several key antibiotics, which indicates that there are components governing the evolution, dissemination and perpetuation of these resistance systems that are yet to be understood.

Resistance to antibiotics can be conferred by chromo-

*Corresponding author. E-mail: saidi_nourhene@yahoo.com. Tel: +21620871718. Fax: +21673664244.

somal or mobile genetic elements (for example, plasmids) and achieved using four main strategies: (i) reduction of membrane permeability to antibiotics; (ii) drug inactivation; (iii) rapid efflux of the antibiotic; and (iv) mutation of cellular target (s) (Krulwich et al., 2005). In addition, antibiotic sequestration has also been suggested as a potential resistance strategy (Pankaj et al., 2009). Overall, the structural and functional characteristics of antibiotic resistance share common themes with those of metal resistance (Baker-Austin et al., 2006).

Although, bacterial exposure to metals predates human history, anthropogenic-derived sources of metals represent a major source of contamination in the environment. Importantly, a substantial number of reports suggest that metal contamination in natural environments could have an important role in the maintenance and proliferation of antibiotic resistance (Alonso et al., 2001; Summers, 2002). This is of particular concern considering that anthropogenic levels of heavy metals are currently several orders of magnitude greater than levels of antibiotics (Stepanauskas et al., 2005). Unlike antibiotics, metals are not subject to degradation and can subsequently represent a long-term selection pressure (Stepanauskas et al., 2005). Thus, there are concerns regarding the potential of metal contamination to maintain a pool of antibiotic-resistance genes in both natural and clinical settings. In addition to metals, other toxicants are implicated in the co-selection of antibiotic resistance, including quaternary ammonium compounds and antifouling agents and detergents (Sidhu et al., 2001; Chapman, 2003).

Several explanations have been proposed for the enhanced resistance of biofilm-associated cells to both metals and antibiotics (Baker-Austin et al., 2006). Both metal and antibiotic sequestration in the biofilm matrix and the presence of a small population of 'persister' cells might be contributing factors in the time-dependent tolerance of both planktonic cells and biofilms to high concentrations of antimicrobial agents (Harrison et al., 2005).

In Tunisia, on the east coast of the country bordering the Mediterranean Sea is a key location for the study of antibiotic resistant bacteria and heavy metals contamination in the aquatic environment. The bay is of great economic importance for fishing and aquaculture of numerous species of crustaceans and fish (Snoussi et al., 2006). In addition, domestic wastes, including industrial wastes are discharged into the bay and the sea.

To our knowledge, our present study is the first to determine the prevalence and resistance to antimicrobial agents and heavy metals of *A. hydrophila* isolated from wastewater, bay, seawater and fish. However, in this work, we focus on the current body of knowledge regar-ding (i) to characterize the *A. hydrophila* strains recovered from Tunisian aquatic biotopes; (ii) to determine the level of antibiotic resistance rates against widely used antibiotics in Tunisia; (iii) to determine the heavy metals resistance of the bacteria; (iv) to investigate the relationship between the antibiotics and heavy metals resistance and (v) to determine the heavy metals effect on *A. hydrophila* slime producing.

MATERIALS AND METHODS

Aeromonas hydrophila strains

This study includes 31 *A. hydrophila* strains: ten strains were isolated from treated wastewater (ONAS), six strains were isolated from the bay of Khenis (Aquaculture center, Monastir), eight strains were isolated from seacoast of Monastir, six strains isolated from ornamental fish and a reference strain *A. hydrophila* ATCC 7966^T [American Type Collection Culture (Manassas, Va.)] (Saidi et al., 2011).

All these strains were identified and characterized by Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and achieved by the conventional methods described by Balows et al. (1991). Gram staining method, cell morphology, the oxidase, catalase, motility (Mannitol-Motility agar, Pronadisa, Madrid, Spain), susceptibility to the vibriostatic compound O/129 (10 and 150 µg/disc) and ampicillin antibiotic (10 µg), growth at 30 and 37°C and growth on Rimler Shotts Agar (mRS) were the first tests employed to identify the organisms belonging to *Aeromonas* genus. Commercial miniaturized strips 20 NE Api (Non *Enterobacteriacae*, bioMerieux, France) were also used.

The production of lipase (Tween 80), haemolysin (Sheep blood agar, Pronadisa, Madrid, Spain) and DNA hydrolysis (DNAse Agar, Sharlau Microbiology, Barcelona, Spain) were tested as described previously by Snoussi et al. (2006). The enzymes amylase and lecithinase were detected on media prepared with phosphate buffer saline (PBS) supplemented, respectively with 0.5% starch and 5% egg yolk emulsion. The caseinase activity was tested according the protocol described by Zanetti et al. (2000). *A. hydrophila* strains were cultured on Nutrient Agar containing 5% skim milk. After incubation for up to 72 h at 37°C, the formation of a clear zone caused by casein degradation was considered as a positive test.

Susceptibility testing

Antibiotic susceptibility was performed according to the national Committee for Clinical Laboratiry Standards (CLSI, 2007) method on Mueller–Hinton Agar (Difco) by the disk-diffusion method (Bauer et al., 1966). Resistance to the following antibiotics (BBL, Md, USA) of *A. hydrophila* strains (10^6 CFU/ml) was tested with disks containing nalidixic acid (NA, 30 µg), tetracycline (TE, 30 µg), gentamicin (GM, 10 µg), imipenem (IPM, 10 µg), neomycin (N, 30 µg), ticarciline (TIC, 75 µg), colistine (CL50, 50 µg), cefoxitine (FOX, 30 µg), cefalotine (CF, 30 µg) and flumequine (UB, 30 µg), oxolinic acid (OA, 10 µg), oxytetracycline (OTC, 30 UI), sulfamide/ trimethoprime (SULF/TMP, 200 µg/5 µg). The strain *A. hydrophila* ATCC 7966^T was used as control.

Multiple antibiotic resistances among *Aeromonas hydrophila*

The multiple antibiotic resistance (MAR) index when applied to a single isolate is defined as ***a/b***, where '***a***' represents the number of antibiotics to which the isolate was resistant and '***b***' represents the number of antibiotics to which the isolate was exposed.

For example if the isolate was exposed to twelve antibiotics and was tolerant to six antibiotics, the index for the isolate would be 6/12 or 0.50 (Liberto et al., 2007). MAR index value higher than 0.2 is considered to have originated from high-risk sources of contamination like human, commercial poultry farms, swine and dairy cattle where antibiotics are very often used. MAR index value of less than or equal to 0.2 is considered the origination of strain from animals in which antibiotics are seldom or never used.

Survival of *Aeromonas hydrophila* under heavy metals concentration

The heavy metals (E-Merck) were used to understand its impact on the growth and survival of *A. hydrophila.* The salts used for the study were Copper sulphate ($CuSO_4.5H_2O$), Cobaltous chloride ($CoCl_2.6H_2O$), Mercuric chloride ($HgCl_2$) and Zinc chloride ($ZnCl_2$).

The tendencies of growth were tested on Trypticase Soy Agar (TSA) medium mixed with different concentrations of heavy metals traces for all *A. hydrophila* strains and the plates were incubated for 24 h at 37°C. The average number on bacteria for every concentration of metal was calculated.

The survival test was also examined by filtering metals using Whatmann filter paper (0.2 µm), and stored at 4°C. From the stock solution, various concentrations like 100, 200, 300, 400, and 500 ppm (Copper, Cobalt and Zinc) and 1, 2, 3, 4 and 5 ppm (Mercury) of metal solutions were prepared and used for the study. Growing *A. hydrophila* strains in sterile nutrient broth at 37°C for 16 h was realized. After, the broth was centrifuged at 12000 rpm for 30 min. The cells were washed with sterile saline solution and transferred into 100 ml phosphate buffer solution and the initial optic density (OD) was taken (Thangavel, 2004). The flasks were kept at 37°C for 24 h and the OD was measured (copper, λ = 480 nm; zinc, λ = 213 nm; cobalt, λ = 425 nm and mercury, λ = 254 nm).

Minimal inhibitory concentration (MIC) of heavy metals

The MIC for each bacterial isolate for heavy metal was determined using Mueller-Hinton agar (Difco) containing heavy metals (Cu^{2+}, Zin^{2+}, and Co^{2+}) at concentrations ranging from 100 to 500 ppm and (Hg^{2+}) at concentrations ranging from 1 to 5 ppm. The isolates were considered tolerant if the MIC values exceeded that of the *Escherichia coli* K-12 strain which was used as the control (Akinbowale et al., 2007).

Slime production on Congo red agar (CRA)

The classic method most often used to phenotypically detect slime production in these bacteria is the Congo red agar (CRA) plate test as described by Freeman et al. (1989). The CRA plate test is performed on a solid medium, the Congo red agar. The direct analysis of the colonies formed on the solid medium allows the recognition of slime-producing strains (characterised by black colonies on the red agar) and of non-slime-producing strains (pink/red coloured colonies).

Congo red agar plate test was prepared by adding 0.8 g/L Congo red (Bio Basic INC) and 36 g of Saccharose (Merck), both of which had been previously autoclaved separately, to 1 L of Brain Heart Infusion Agar (Scharlau Microbiology, Pronadisa, Madrid, Spain). Plates were incubated for 24 h at 37°C and subsequently overnight at room temperature (Freeman et al., 1989). Slime-producing *A. hydrophila* grew as black colonies, while non slime-producing strains grew as red colonies. The original test was optimized by using a colorimetric scale with six tonalities: very black, black and approximately black were considered as positive results, while burgundy, red and very red were considered as negative results (Subashkumar et al., 2006). *Staphylococcus aureus* ATCC 25923 was used as positive control for slime production and *Staphylococcus epidermidis* CIP 106510 was used as negative control (Chaieb et al., 2007).

Aeromonas hydrophila morphology visualization by atomic force microscopy

To visualize the bacteria after heavy metal exposure on glass slides and to have an idea on the morphological changes in the cells during heavy metal stress, *A. hydrophila* ATCC 7966^T cells was used as a negative control. For the experiments, the cells enriched on PBS with different concentrations of mercury (1, 2, 3, 4 and 5 ppm) were collected, washed three times by PBS, centrifuged and the pellet was resuspended in PBS, fixed on a sterilized round microscope cover slide and the piece was examined by Atomic force Microscope (AFM, Nanoscope IIIA, Digital Instrument; Veeco) according to the method previously described (Braga and Ricci, 1998).

Statistical analysis

All results are shown as the average of at least three independent experiments; variation is expressed as standard deviation. The Pearson correlation coefficient was calculated to determine the possible relation between the resistance to heavy metals and the resistance to antibiotics. All statistics were performed using SPSS for Windows version 17.0.

RESULTS

Antibacterial resistance

The identified strains were multi-resistant to various antibiotics used including those exploited in the treatment of *Aeromonas* disease of the fish (flumequine, oxolinic acid, sulfamide + trimetoprime and oxytetracycline). Indeed, all bacteria were sensitive to gentamicin.

The results showed that bay, treated waste water *A. hydrophila* isolates were more resistant to almost tested antibiotics than sea water and fish *A. hydrophila* (nalidixic acid (70 and 60%), ticarcyline (60 and 50%), cefoxitine and cefalotine (100 and 90%)), respectively. While the isolates of seawater were most sensitive to the majority of antibiotics, all strains were sensitive for neomycin, tetracycline, fumequine, oxilinic acid, oxytetracycline and sulfamide-trimethoprime.

On the other hand, *A. hydrophila* strains isolated from moribund fish were fairly tolerant to certain antibiotics (colistine (50%), nalidixic acid and cefalotine (66.66%)) and completely sensitive to the oxilinic acid and sulfamide + trimethoprime (Figure 1). The study of the MAR index of these 31 isolates showed that these bacteria presented a high risk, indeed, the recorded values were higher than 0.2, what corresponded to 74.19% of the studied stocks (Table 1).

Aeromonas hydrophila resistance to heavy metals effects

In the present study, resistance to copper (Cu^{2+}), cobalt (Co^{2+}), zinc (Zn^{2+}) and mercury (Hg^{2+}) were studied for all the isolates. In the four sample types (treated waste water, bay, sea water and fish), resistance to heavy metals was described in the Table 2. Actually, all *A. hydrophila* are tolerant to various heavy metals tested and they presented a tolerance reaching 300 ppm (copper, zinc and cobalt) and 3 ppm (mercury). For a concentration reaching 400 ppm, all the strains were tolerant to copper,

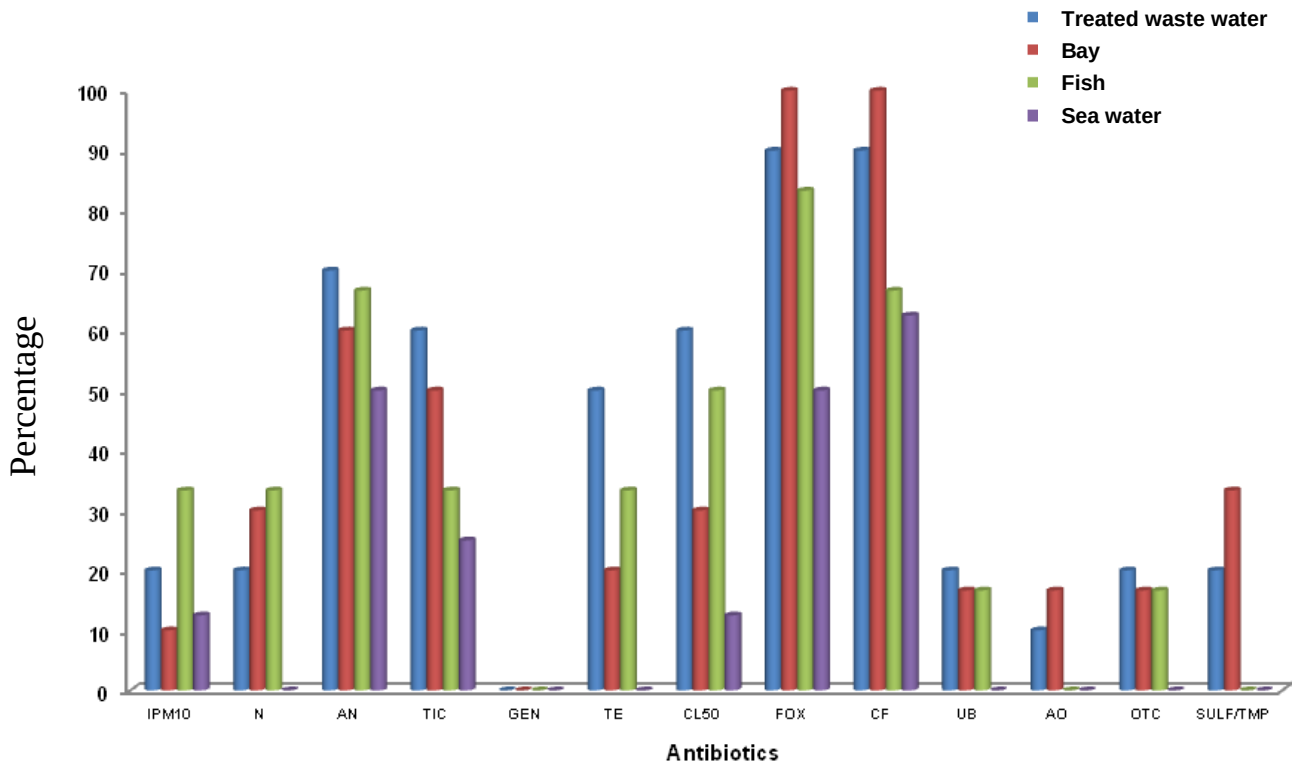


Figure 1. Percentage of resistance to 13 antibiotics of the 31 *A. hydrophila* strains isolated from treated wastewater, sea water, bay and fish. Antibiotics tested are as follow: **IMP**: Imipenem (10 µg), **N**: Neomycin (30 µg), **NA**: Nalidixic acid (30 µg), **TIC**: Ticarcilline (75 µg), **GEN**: Gentamicin (10 UI), **TE**: Tetracycline (30 µg), **CL50**: Colistine (50 µg), **FOX**: Cefoxitine (30 µg), **CF**: Cefalotine (30 µg), **UB**: Flumequine (30 µg), **OA**: Oxolinic acid (10 µg), **OTC**: Oxytetracycline (30 UI), **SULF/TMP**: Sulfamide/Trimethoprime (200 µg/5 µg).

zinc and cobalt, but, only 10% of the isolates from treated wastewater and bay were tolerant to mercury (4 ppm).

The higher tolerance of *A. hydrophila* to 500 and 5 ppm concentrations of various metals traces had proven to be significant for the isolates of bay (100, 83.33, 66.66 and 10%) and of treated wastewater (60, 70, 50 and 10%), respectively for copper, zinc, cobalt and mercury. Whereas the small percentages of resistance were detected in the isolates of sea water (25, 37.5, 0 and 0%) and of the fish (25, 10, 0 and 0%), respectively for same metals quoted previously.

The Table 3 described the viability of *A. hydrophila* continuation of the different concentration effect from selected heavy metals. Indeed, for a concentration of 500 ppm copper, the number of the viable bacteria arrived at 5.01×10^4 CFU/ml (treated wastewater), 7.22×10^4 CFU/ml (bay), 5.16×10^2 CFU/ml (sea water) and 5×10^2 CFU/ml (fish), which was equivalent to 0.492, 0.43, 0.482 and 0.49 of OD, respectively. However, the less significant results were recorded for zinc and cobalt. On the other hand, at 5 ppm of mercury concentration, only the isolates of treated wastewater and bay presented viability up to 1.24 and 1.5 CFU/ml, corresponding to 0.043 and 0.023 OD.

Atomic force Micrography of the bacteria morphology (Figures 2a-b) showed that *A. hydrophila* after the mercury exposure (5 ppm), have changed form and become coccoid. These morphological modifications allow the adaptation to mercury stress.

Slime production under heavy metals

The objective to determine the effect of the tested metal on the slime production in isolated *A. hydrophila*, we found that after exposure to mercury, 3/10 of the treated wastewater isolates, 2/8 of sea water strains and only one strain from fish changed their phenotypical profile and became non slime producer and thereafter produced new morphotype (brown, pink and orange colonies). On the other hand, *A. hydrophila* of bay and those isolated from moribund fish did not modify their morphotype (Figure 3).

The Table 4 showed the resistance of all isolates to the effects of copper, zinc and cobalt and no morphotypic modification was registered in bay case. Similar results were found in the case of the treated wastewater isolates except for case of cobalt; indeed, only one strain changed profile and became non slime producer.

Whereas, *A. hydrophila* isolated from fish and sea water presented the most significant modifications, indeed, for copper, zinc and cobalt the percentages of morphotype

Table 1. MAR Index and Model of resistance of *the A. hydrophila*.

Strain	MAR index	Model of antibiotics resistance
Treated wastewater		
WT1	0.33	AN-TIC-FOX- SULF/TMP
WT2	0.41	IMP-AN-TIC-FOX -SULF/TMP
WT3	0.16	AN-FOX
WT4	0.16	TE -UB
WT5	0.33	TE-CL50-FOX- AO
WT6	0.33	AN-TIC-CL50-FOX
WT7	0.5	IMP-AN-TIC-CL50-FOX -OTC
WT8	0.5	AN-TIC-TE-CL50-FOX -OTC
WT9	0.58	N-AN-TIC-TE-CL50-FOX-UB
WT10	0.33	N-TIC-CL50-FOX
Bay		
R1	0.33	IMP-TIC-TE-FOX
R2	0.25	AN-TIC-FOX
R3	0.41	AN-CL50-FOX -AO-SULF/TMP
R4	0.33	AN-CL50-FOX -UB
R5	0.25	N-AN-FOX
R6	0.41	TIC-CL50-FOX- OTC-SULF/TMP
Fish		
E2	0.25	AN-FOX -UB
E3	0.5	AN-FOX -UB-AO-OTC-SULF/TMP
E4	0.33	N-TIC-FOX-OTC
E5	0.5	IMP-AN-TIC-TE-CL50-FOX
E6	0.33	IMP-N-AN-CL50
E7	0.66	IMP-N-AN-TIC-GEN-TE-CL50-FOX
Sea water		
S1	0.08	CL50
S2	0.5	AN-FOX -UB-AO-OTC-SULF/TMP
S3	0.66	IMP-N-AN-TIC-GEN-TE-CL50-FOX-CF
S4	0.08	FOX
S5	-	-
S6	0.08	AN
S7	0.16	AN-TIC
S8	0.16	AN-FOX

IMP: Imipenem. **N**: Neomycin. **AN**: Nalidixic acid. **TIC**: Ticarcilline. **GEN**: Gentamicin. **TE**: Tetracycline. **CL50**: Colistine. **FOX**: Cefoxitine. **UB**: Flumequine. **AO**: Oxolinic acid. **OTC**: Oxytetracycline. **SULF/TMP**: Sulfamide/Trimethoprime.

modification of the sea water isolates were 1/8, 2/8 and 2/8, respectively. On the other hand, these values were 0, 1/6 and 2/6 for *A. hydrophila* isolated from fish.

DISCUSSION

The *Aeromonas hydrophila* resistance to antibiotics

The study of antibiotic resistance in water organisms is important, as it might indicate the extent of alteration of water ecosystems by human action. Actually, water bacteria could be indigenous to aquatic environments, or exogenous, transiently and occasionally present in the water as a result of shedding from animal, vegetal, or soil surfaces.

According to our results, bay and treated waste water *A. hydrophila* isolates were more tolerant to almost tested antibiotics than sea water and fish *A. hydrophila*. Martinez (2003) has found similar results and he has shown that more than 90% of bacterial strains originated from sea-water are tolerant to more than one antibiotic, and 20% are tolerant at least to five. The resistance of the strains to

Table 2. Tolerance of *A. hydrophila* isolated from treated wastewater. fish. bay and sea water to heavy metals.

Metal/environment	N	Heavy metal concentrations (ppm)				
		1*/100	2*/200	3*/300	4*/400	5*/500
Copper						
Treated wastewater	10	100	100	100	100	60
Sea water	08	100	100	100	100	25
Bay	06	100	100	100	100	100
Fish	06	100	100	100	100	25
Zinc						
Treated wastewater	10	100	100	100	100	70
Sea water	08	100	100	100	100	37.5
Bay	06	100	100	100	100	83.33
Fish	06	100	100	100	100	10
Cobalt						
Treated wastewater	10	100	100	100	100	50
Sea water	08	100	100	100	100	0
Bay	06	100	100	100	100	66.66
Fish	06	100	100	100	100	0
Mercury*						
Treated wastewater	10	100	100	100	10	10
Sea water	08	100	100	100	0	0
Bay	06	100	100	100	10	10
Fish	06	100	100	100	0	0

Reference of test: Minimal Inhibiting Concentration of the standard strain *Escherichia coli* K12.

antibiotics could be explained by the possibility of the heavy use of these compounds in aquaculture (bay), several of which are non biodegradable increases antibiotic selective pressure in water, facilitates the transfer of antibiotic resistance determinants between aquatic bacteria, including fish and human pathogens, and allows the presence of residual antibiotics in commercialized fish and shellfish products (Rhodes et al., 2000; Cabello, 2006). Antibiotic residues entering this aquatic environment from different sources may increase the distribution of potential drug-resistant pathogen bacteria (Matyara et al., 2008). However, some studies indicate that increasing heavy metal concentrations lead to a decrease of antibiotic resistance (Stepanauskas et al., 2005; McArthur and Tuckfield, 2008; Hölzel et al., 2012). The secontradicting results were investigated by Hölzel et al. (2012). In consequence of the addition of mercury chloride ($HgCl_2$) to the antimicrobial test procedure, the MIC for a wide range of antibiotics decreased. This observation could be due to an interaction of Hg with enzymes or nucleic acids which cause antibiotic resistance. $HgCl_2$ could also have acotoxic effect with antibiotics that interfere with ribosomes because the generation of the Hg-degraded enzyme would be inhibited. Furthermore, Hölzel et al. (2012) mentioned also a possible metal induced shift within the bacterial community to ward Hg tolerant bacteria where by the benefit of antibiotic resistance in the presence of antibiotics would be out competed. The increased antibiotic susceptibility in consequence of Hg exposure could also play a role in the observations of other field studies (Seiler and Berendonk, 2012).

Multiple antibiotic resistance (MAR) index of *Aeromonas hydrophila*

Like Gram negative bacilli, the emergence of resistance among Aeromonads will be accelerated by the extensive clinical use of antibiotics (Ko and Chung, 1995; Chaudhury et al., 1996). Such high level of multiple drug resistance may arise from selective pressure due to the indiscriminate use of antibiotics. The variation in the drug resistance may be related to the source of *A. hydrophila* isolated and the frequency prescribed for treating *Aeromonas* infections in geographical areas (Radu et al., 1997).

These reports revealed that geographical, socio economical parameters and local selective pressures could influence antibiotic resistance among *Aeromonas* spp. Growing incidence of MAR among *A. hydrophila* strains isolated from various sources has been reported from many parts of the world (Radu et al., 2003). In our study,

Table 3. The viability of *A. hydrophila* isolated from the treated wastewater; sick and healthy fish; bay and sea water of ornamental fish after the heavy metals effect.

Metal element	Concentration (ppm)	CFU/ml ±SD				OD* (A)			
		Treated waste water	Sea water	Bay	Fish	Treated wastewater	Sea water	Bay	Fish
Copper	100	$35.00 \pm 1.37 \times 10^4$	$29.76 \pm 0.36 \times 10^2$	$65.64 \pm 2.75 \times 10^4$	$26 \pm 1.15 \times 10^2$	0.211	0.231	0.153	0.18
	200	$25.38 \pm 0.69 \times 10^4$	$22.3 \pm 1.00 \times 10^2$	$52.39 \pm 0.91 \times 10^4$	$17 \pm 1.15 \times 10^2$	0.261	0.295	0.21	0.311
	300	$19.45 \pm 0.43 \times 10^4$	$15.65 \pm 0.70 \times 10^2$	$35.22 \pm 0.60 \times 10^4$	$11.5 \pm 1.73 \times 10^2$	0.334	0.315	0.277	0.323
	400	$12.36 \pm 0.91 \times 10^4$	$10.41 \pm 0.34 \times 10^2$	$22.27 \pm 0.94 \times 10^4$	$6.5 \pm 0.57 \times 10^2$	0.414	0.398	0.36	0.4
	500	$5.01 \pm 0.50 \times 10^4$	$5.16 \pm 0.50 \times 10^2$	$7.22 \pm 0.36 \times 10^4$	$5 \pm 1.15 \times 10^2$	0.492	0.482	0.43	0.49
Cobalt	100	$26.68 \pm 0.97 \times 10^4$	$24.86 \pm 0.51 \times 10^2$	$56.72 \pm 0.25 \times 10^4$	$20.5 \pm 0.57 \times 10^2$	0.221	0.201	0.113	0.212
	200	$16.48 \pm 0.43 \times 10^4$	$20.8 \pm 0.64 \times 10^2$	$42.3 \pm 0.62 \times 10^4$	$14.5 \pm 1.73 \times 10^2$	0.225	0.261	0.221	0.267
	300	$15.58 \pm 0.24 \times 10^4$	$14.31 \pm 0.88 \times 10^2$	$27.4 \pm 0.64 \times 10^4$	$12.5 \pm 1.73 \times 10^2$	0.305	0.324	0.287	0.331
	400	$10.83 \pm 0.68 \times 10^4$	$9.41 \pm 0.46 \times 10^2$	$19.65 \pm 0.32 \times 10^4$	$5 \pm 1.15 \times 10^2$	0.298	0.274	0.3	0.288
	500	$4.63 \pm 0.42 \times 10^4$	0 ± 0.0	$4.76 \pm 0.90 \times 10^4$	0 ± 0.0	0.322	0.394	0.2	0.411
Zinc	100	$30.44 \pm 0.63 \times 10^4$	$19.65 \pm 0.34 \times 10^2$	$36.17 \pm 0.80 \times 10^4$	$15 \pm 1.15 \times 10^2$	0.103	0.2	0.1	0.231
	200	$21.16 \pm 0.98 \times 10^4$	$15.33 \pm 0.68 \times 10^2$	$21.21 \pm 0.92 \times 10^4$	$10.5 \pm 0.57 \times 10^2$	0.141	0.291	0.125	0.322
	300	$16.49 \pm 0.53 \times 10^4$	$13.45 \pm 0.88 \times 10^2$	$17.92 \pm 0.13 \times 10^4$	$8.5 \pm 0.57 \times 10^2$	0.177	0.304	0.215	0.331
	400	$11.06 \pm 0.10 \times 10^4$	$7.63 \pm 0.39 \times 10^2$	$10.81 \pm 0.28 \times 10^4$	$3.5 \pm 0.57 \times 10^2$	0.26	0.414	0.208	0.425
	500	$3.41 \pm 0.46 \times 10^4$	$1.9 \pm 0.15 \times 10^2$	$4.42 \pm 0.21 \times 10^4$	$1 \pm 0.0 \times 10^2$	0.3	0.462	0.3	0.485
Mercury	1	$9.65 \pm 0.48 \times 10^2$	$6.65 \pm 0.48 \times 10^2$	$15.44 \pm 0.67 \times 10^2$	$2.5 \pm 0.57 \times 10^2$	0.143	0.199	0.11	0.211
	2	$7.92 \pm 0.97 \times 10^2$	6.5 ± 0.44	$13.06 \pm 0.48 \times 10^2$	2 ± 0.0	0.11	0.23	0.1	0.235
	3	$1.66 \pm 0.51 \times 10^2$	2 ± 0.0	$9.96 \pm 0.76 \times 10^2$	2 ± 0.0	0.107	0.322	0.055	0.342
	4	$3.5 \pm 0.57 \times 10^2$	0 ± 0.0	$3.62 \pm 0.48 \times 10^2$	0 ± 0.0	0.073	0.431	0.043	0.445
	5	1.24 ± 0.21	0 ± 0.0	1.5 ± 0.57	0 ± 0.0	0.043	0.452	0.023	0.463

*: λ = 480 nm (copper). λ = 213 nm (zinc). λ = 425 nm (cobalt) and λ = 254 nm (mercury).

the MAR index of the 31 isolates ranged between 0.08 and 0.66. Hence, almost all isolates were from the high risk source contamination like faecal-oral contamination. Due to indiscriminate use of antibiotics, the microorganisms might have developed resistance towards several antibiotics. Under these circumstances, it will be worth while to find out prevalence of antibiotic resistance of *A. hydrophila* strains that may be considered as an emerging pathogen and to identify the high-risk source. Indeed, multidrug resistant pathogens are the serious problem nowadays faced by the clinicians.

Such a multiple antibiotic resistant strains enter the community, and hybridize with non-MAR strains resulting in the transfer of resistant plasmids and become a serious problem in controlling these strains.

Co-resistance of antibiotic and metal-resistance traits

There is growing concern that metal contamination functions as a selective agent in the proliferation of antibiotic resistance. Documented associations between the types and levels of metal contamination and specific patterns of antibiotic resistance suggest that several mechanisms

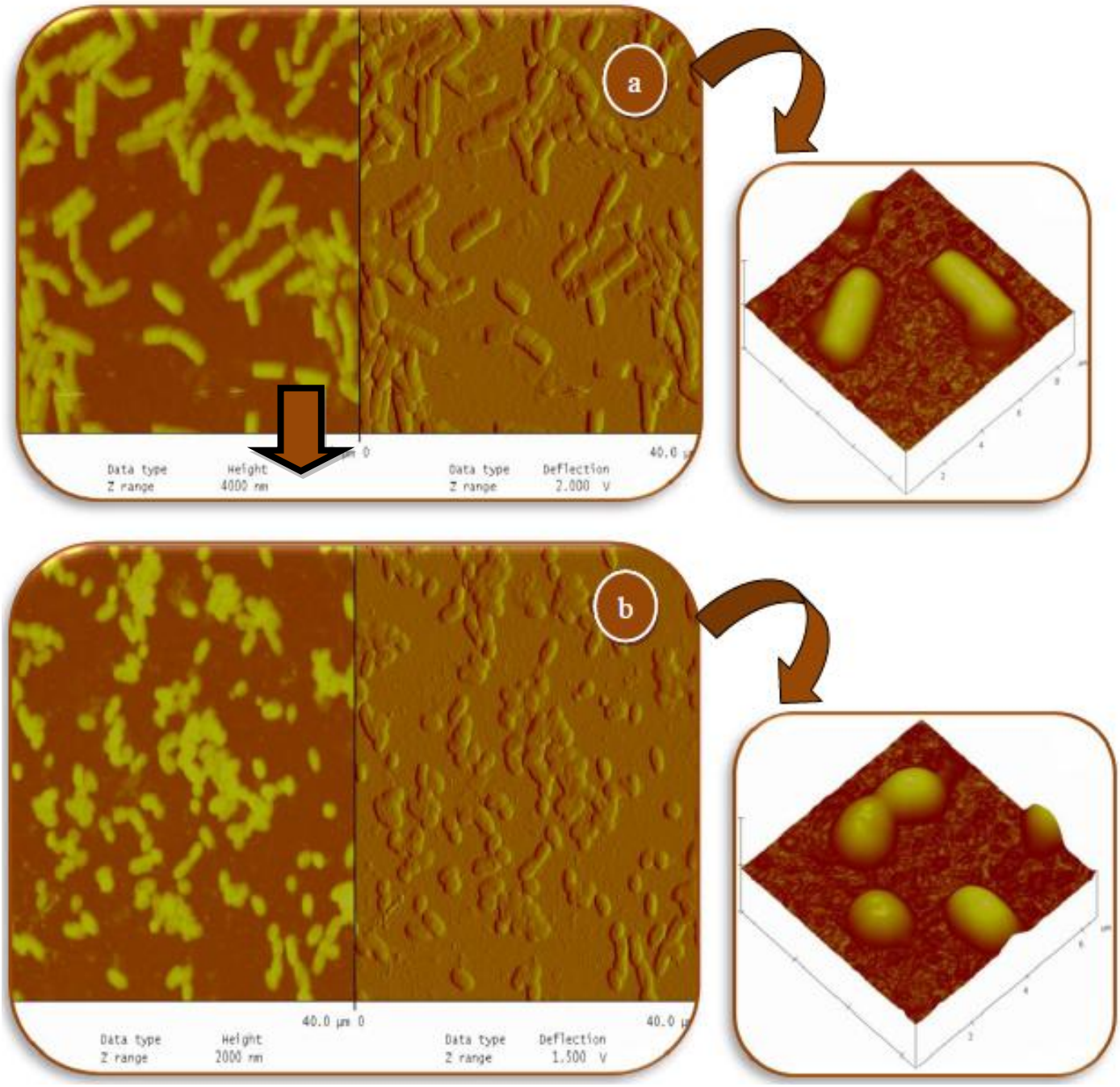
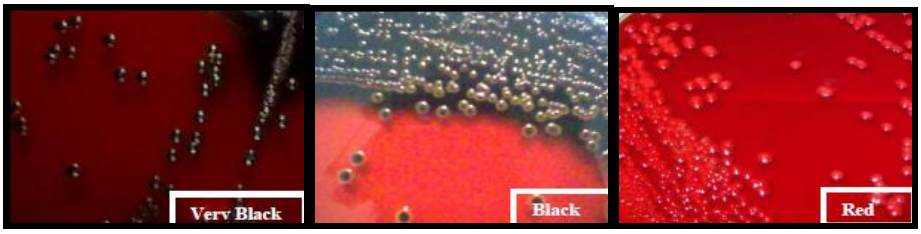


Figure 2. Morphological modification of *A. hydrophila* examined by Atomic force Microscope (AFM): (a) bacillus form to (b) coccoid form after mercury exposure.

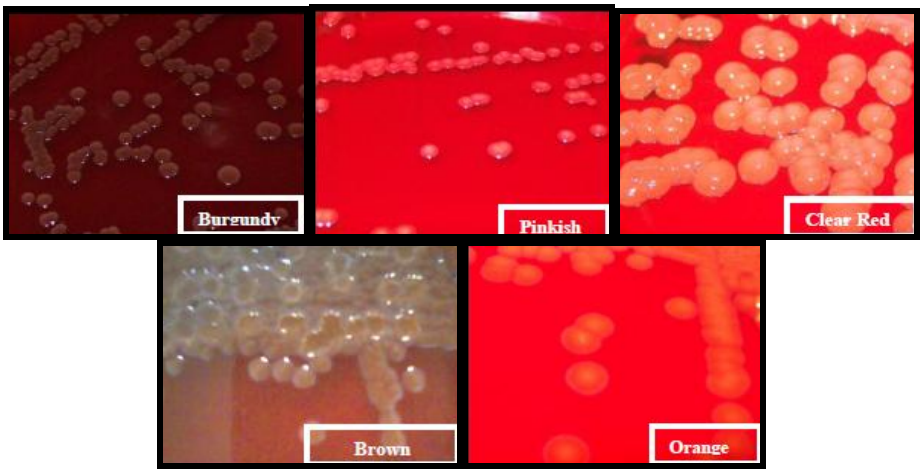
underlie this co-selection process. These co-selection mechanisms include co-resistance (different resistance determinants present on the same genetic element) and cross-resistance (the same genetic determinant responsible for resistance to antibiotics and metals) (Clutterbuck et al., 2007).

Our results revealed that the wastewater and bay isolates were more tolerant to heavy metals (copper, zinc, cobalt and mercury) than sea water and fish strains; similar results of resistances have been shown for antibiotics.

The association of antibiotic-resistance and resistance to heavy metals is very frequent in the same organism (also in the same plasmid, transposon, or integron) so that industrial pollution probably selects for antibiotic-resistance and vice versa (Baker-Austin et al., 2006). The studies of Seiler and Berendonk (2012) have investigated the co-selection in the environment and they showed the presence of correlation between increased heavy metal



(a) Before heavy metal exposure



(b) After heavy metal exposure

Figure 3. Morphotypes of *A. hydrophila* based on the colorimetric scale obtained on Congo red agar before heavy metals exposure **(a):** Very Black colonies (A. *hydrophila* ATCC7966); Black colonies; Red colonies and after heavy metals exposure **(b):** Brown colonies; Burgundy colonies; Orange colonies and Pinkish colonies. *Staphylococcus epidermidis* producing and non-producing slime were used as negative and positive controls.

concentrations with increased phenotypic or genotypic antibiotic resistance. In addition, others researches proved that metal contamination represent a long-standing, widespread, and recalcitrant selection pressure for multi-resistant organisms (Pathak and Gopal, 2005). For the non aquatic organisms, obviously the density of antibiotic - resistance organisms and antibiotic-resistance genes in fresh water varies with the proximity to areas with increased antibiotic consumption, metal pollution, and between seasons, being more frequently found in rainy seasons (Peak et al., 2007).

Evidence for co-selection of antibiotic and metal resistance in the environment originates from diverse habitats contaminated with a variety of metals, which indicates that co-selection is not limited to a subset of metals, environments or microbial taxonomic groups. The strength of evidence presented by these studies ranges considerably between anecdotal reports of co-resistances to experimental studies that unambiguously implicate metals in antibiotic resistance co-selection (Baker-Austin et al., 2006).

Actually, the results of this work has proven that *A. hydrophila* isolated from fish were tolerant to high concentration of heavy metals (copper, zinc and cobalt (100 to 400 ppm) and mercury (3 ppm)). Although these studies do not directly address the hypothesis that metal exposure co-selects for antibiotic resistance, they highlight the fact that metal and antibiotic resistances are commonly found within the same bacteria. Indeed, potential public health concerns for the co-resistance of metal and antibiotic resistances were raised by Pathak and Gopal (2005), who observed that bacterial isolates obtained from fish tissue commonly consumed by humans exhibited resistance to multiple metals and antibiotics.

As evidenced in previous studies, subsequent exposure to elements of heavy metal leads to direct selection for metal-resistance while co-selection for antibiotic resistance. Maintenance of the co-selection antibiotic resistance was accomplished by co-resistance, cross resistance, and co-regulation of the resistant genes (Miranda and Castillo, 1998; Spain and Alm, 2003; Stepanauskas et al., 2005; Wright et al., 2006).

Nonetheless, bacterial resistance to heavy metal was

Table 4. The effect of heavy metals on the slime production of *A. hydrophila* isolated from treated wastewater, sea water, bay and fish.

Origin/metal	N	Slime production (%)	
		Before	After
Copper			
Treated wastewater	10	07/10	07/10
Bay	06	03/06	03/06
Sea water	08	04/06	03/06
Fish	06	06/06	06/06
Zinc			
Treated wastewater	10	07/10	07/10
Bay	06	03/06	03/06
Sea water	08	04/08	02/08
Fish	06	06/06	05/06
Cobalt			
Treated wastewater	10	07/10	06/10
Bay	06	03/06	03/06
Sea water	08	04/08	02/08
Fish	06	06/06	04/06
Mercury			
Treated wastewater	10	07/10	04/10
Bay	06	03/06	03/06
Sea water	08	04/08	02/08
Fish	06	06/06	05/06

emphasized in the present study because substantial number of reports have been alerting on maintenance and proliferation of antibiotic resistance. Resistance genes to both substances were presumably residing closely on the bacterial plasmid and transported together in the environment (Sabry et al., 1997; Spain and Alm, 2003; Wright et al., 2006).

Interaction between antibiotics, heavy metals resistance and slime production in *A. hydrophila*

Many bacteria in the environment exist in surface-attached communities; in fact, the initial bacterial monolayer adhering to polymeric surfaces is converted to a typical biofilm consisting of bacteria plus an extracellular substance (Heilmann et al., 1996). As compared with planktonic bacteria, biofilm bacteria are more tolerant to several antimicrobial agents or other environmental stresses. It has been postulated that large amounts of biofilm formed by these microorganisms play an important role in the degradation and transformation of pollutants in the increasingly polluted soil and water environment (Meng-Ying et al., 2009).

Moreover, biofilm bacteria are usually embedded in an extracellular polymeric substance (EPS) matrix composed of polysaccharides, proteins, and nucleic acids (Whitfield, 1988; Flemming and Wingender, 2001; Sutherland, 2001; Whitchurch et al., 2002). Furthermore, the production of this nature substance termed “slime” appears to play a relevant role (Cristhensen et al., 1982; An and Friedmann, 1998).

The result of this work has shown that the antibiotic and heavy metals resistant strains were biofilm positive and producing slime on CRA. Besides, *A. hydrophila* isolated from treated wastewater and bay has presented the important viability under heavy metals effect than fish and sea water strains. Therefore, these findings were confirmed by Liberto et al. (2007), these researchers have shown that adhesion, bacterial proliferation and slime production increase antibiotic resistance, since drugs may not be able to reach bacteria kept in rein in biofilm.

Further, this work proved the importance of the function played by slime to protect the water environment from selective events caused by the antibiotic and heavy metal release and reduced antibiotic susceptibility, which are acting more effectively on planktonic bacteria (Baquero et al., 2008) and proved that the degree of penetration is dependent on the biofilm and the antimicrobial agent. Clutterbuck et al. (2007) have demonstrated that EPS also can act as an ion exchange and is able to sequester hydrophobic and positively charged antibiotics such as aminoglycosides. On the other hand, Teitzel and Parsek (2003) have suggested that bacteria have developed a variety of resistance mechanisms to counteract heavy

metal stress. These mechanisms include the formation and sequestration of heavy metals in complexes, reduction of a metal to a less toxic species, and direct efflux of a metal out of the cell (Outten et al., 2000).

A proposed mechanism that contributes to this increased resistance is binding and sequestration of antimicrobial agents by EPS components, such as negatively charged phosphate, sulphate, and carboxylic acid groups (Hunt, 1986). Another factor that may contribute to the resistance of biofilms is that many antimicrobial agents target metabolically active cells (Teitzel and Parsek, 2003). However, slime production and association in biofilm are two parameters of great complexity; they are highly correlated with the environment.

The present study focuses on a part of the northern Mediterranean region, wastewater, bay and seawater samples from a polluted aquatic environment. It was established that there is a possible association between heavy antimicrobial consumption within a population and the frequent recovery of antibiotic resistant bacteria. However, it is apparent that a range of other agents might represent important mechanisms that drive the selection of antibiotic-resistance determinants.

Current advances in microbial genomics, physiology and biochemistry could provide the basis for the precise determination of important processes involved in metal–antibiotic resistance interactions. Areas of particular interest include the multifunctional properties of co-resistance determinants and the relative contributions of these resistance systems to the fitness of bacteria in different environmental and clinical settings. It is necessary to evaluate potential mechanisms at several levels of biological organization to comprehensively assess the role of metal contaminants as a selective force in maintaining and propagating the pool of antibiotic-resistance determinants in the environment.

The geographic scope of this study should include other parts of the Tunisian coasts on the Mediterranean Sea. Furthermore, more studies should be developed - cheap and reliable: first, bacterial clones and resistance genes source tracking; second, detection of antibiotics in water environments; third, identification of the mechanisms involved in the association between antibiotic, metal resistances and slime producing, fourth, disinfection of water from antibiotic-metal-resistant populations and the resistance gene pool, and removal of antibiotics from wastewater.

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Full Length Research Paper

A new bioassay using *Chlorella vulgaris* cell density for detecting mycotoxins

M. B. Aboul-Nasr[1]*, Abdel-Naser A. Zohri[2] and Enas Mahmoud Amer[2]

[1]Department of Botany, Faculty of Science, University of Sohag, Egypt.
[2]Department of Botany and Microbiology, Faculty of Science, University of Assiut, Egypt.

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Bioassay is an alternative screening technique to evaluate the biotoxicity of the fungal secondary metabolites before any further chemical analyses. In this study, the cell density of *Chlorella vulgaris* cultures were used to detect the biotoxicity of 108 isolates belonging to seven fungal species. The crude extracts of all toxin producing fungal isolates that were tested inhibited the growth of *C. vulgaris* dramatically after 48 and 72 h as compared to the control. The crude extracts of the nontoxin producing fungal isolates that were tested showed that there was no significant inhibition of *C. vulgaris* at the three time intervals as compared to the control. These results corresponded to the *Bacillus subtilis* disk diffusion method. The use of optical density readings of the growth of *C. vulgaris* reflected a rapid, easy and effective tool to detect mycotoxins.

Key words: Bioassay, *Chlorella*, colorimetric, mycotoxins, pathogenicity.

INTRODUCTION

Mycotoxins are an extremely diverse group of biological compounds of secondary fungal metabolites with low molecular weight (mostly below 700 Da). The chemical structure and physical properties of these toxins are widely varied. When these toxins are ingested, inhaled or absorbed through the skin, they cause lowered performance, sickness or death in man or animals and birds (Van Egmond and Speijers, 1999).

Biological assay is the determination of the relative strength of a substance by comparing its effect on a test organism with that of a standard preparation. It has become increasingly useful for mycotoxins detection as a precursor to chemical analysis. Bioassay allows the analyst to make an informed decision when selecting a more detailed chemical analysis procedure. Various short-term *in vitro* biological assays have been employed to screen for the presence of several mycotoxins (Lompe and Milczewski, 1979; Coker, 1997). Problems encountered with these bioassays include the maintenance of animals or cell lines and cultures, technically complex procedures requiring extensive preparation or assay times or both, expensive materials, and subjective data analyses (Blaise, 1991) also. Recent toxicology, in accordance with recommendations from the European Council, has demanded decrease in the number of vertebrates used in toxicology testing and their partial replacement with invertebrate animals, plants or even organ, tissue, or cell cultures (Petr Dvorak et al., 2012). During the last 50 years, various invertebrate species have been tested for their sensitivity to many chemical or physical agents to prove their possible use for pre-screening tests. Brine shrimp larvae have been used to evaluate fungal toxins (Harwig and Scott, 1971). The lower sensitivity of the *Artemia* species to several chemical or physical agents in comparison with the other invertebrate test organisms, the decreased solubility of some chemical substances in saline or sea

*Corresponding author. E-mail: mbassamaboulnasr@yahoo.com. Tel: +201003459776. Fax: +20882288844.

medium, in addition to the various conditions that control the test, such as temperature, pH, chemical composition of the medium, oxygen, photoperiod, nutrients, some population effects, type of growth stage, etc may affect the toxicity results (Nalecz-Jawecki et al., 2003; George-Ares et al., 2003; Mayorga et al., 2010). Algae are especially suitable for biotests because of their sensitivity to environmental pollution and their abundance in aquatic systems. In addition, they have no roots as higher plants and reflect only the properties of the ambient water rather than those of the soil higher plants that are rooted. Also, algal biotests are simple and allow observing multiple generations (Danilov and Ekelund, 2000). The agar diffusion technique of *Chlorella vulgaris, Ustilago maydize* and *Trichoderma viride* used by Bean et al. (1992) showed that *Chlorella* was the most sensitive organism toward macrocyclic trichothecens produced by different *Myrothecium* species tested. *Bacillus subtilis* and *C. vulgaris* were proved to be particularly sensitive to mycotoxins (Sukroongreeung et al., 1984).

This study aimed to develop a rapid, easy, sensitive and reliable bioassay technique using optical density readings of liquid cultures of *C. vulgaris* to detect mycotoxic compounds.

MATERIALS AND METHODES

Fungal isolates

One hundred and eight isolates of filamentous fungi isolated from the air of intensive care units and operation rooms as well as the dust of air conditioning system filters in each of the Assiut University hospitals were collected and identified in Assiut University Mycological Center. Multiple of six isolates of each *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Cladosporium cladosporioides*, *Fusarium solani*, *Fusarium oxysporum* and *Stachybotrys elegans* were tested.

Fungal crude extracts

Each isolate was grown on Czapek's glucose agar medium under aseptic conditions, incubated at 25±2°C for 10 days. After the incubation period, the entire agar media with fungal mycelia were cut into small pieces, transferred to a 250 ml Erlenmeyer flask containing 50 ml 96% methanol. The contents were shaken on a rotary shaker (200 rpm, 24 h) and filtered through filter paper (Whatman No.1). The residue was then washed twice with 96% methanol (25 ml each). The methanol extracts were combined, dried over anhydrous sodium sulphate, and then left to evaporate to near dryness under vacuum. The residues were transferred quantitatively to a dram vial with the minimal amount of methanol and evaporated to near dryness (Bean et al., 1992).

Thin layer chromatographic (TLC) determination of mycotoxins

For qualitative determination of mycotoxin produced by different fungal isolates tested, TLC technique adopted by El- kady and Moubasher (1982) was employed.

Biological assay procedure

Bacillus subtilis

The disc diffusion method (Sleigh and Timburg, 1981) was used to measure the antibacterial activities of different isolates crude extract on *B. subtilis* and the inhibition zone was measured.

Chlorella vulgaris

The strain of *C. vulgaris* used in this investigation was obtained from Laboratory of Microbiology, Department of Botany and Microbiology, University of Assiut. For growth and enrichment, Beijrinek medium was used (Stein, 1966). Algal cultures were grown at a temperature of 28 ±1°C in a light growth chamber (Forma Scientific, USA). The inoculums were maintained to be 0.123 O.D. 750 nm in all the cultures throughout the study period. Three replicates of 10 µl of the clean crude extract tubes and 6 ml of *Chlorella* culture was added to each tube under a septic conditions, optical density (O.D. 750 nm) of cultures was measured at required time intervals (after 24, 48 and 72 h) using spectrophotometer Thermo scientific, evolution 160 UV-Vis, double beam spectrophotometer, USA.

Data analysis

The results were analysed by one way analysis of variance (ANOVA) followed by Newman-Keuls Multiple Comparison Test as a post-test using computer prism program for windows, version 3.0 (Graph pad software, Inc, San Diago CA. USA). The significant difference between the tested groups was accepted at $p<0.05$, 0.01 or 0.001, the data were expressed as mean ± standard error (SE) and the number of isolates (n) was the multiples of six.

RESULTS AND DISCUSSION

The TLC analysis of the 108 crude extracts showed that only 78 of the fungal isolates that were tested had the ability to produce at least one of these mycotoxic compounds (Aflatoxins B_1, B_2, G_1, G_2 Gliotoxin, fumigillin, T_2, zearalenone, Roridins A and E, verrucarins A and J, Trichoveroids, Satratoxins H and E).

The crude extracts of all toxin producing fungal isolates that were tested according to the TLC analysis inhibited the growth of *C. vulgaris* dramatically after 48 and 72 h of which *F. oxysporum* and *S. elegans* were the fungal isolates that affected the growth of *C. vulgaris* the most as compared to the control (Figure 1B and C). Although, the growth of *C. vulgaris* was significantly affected after 24 h, the most fungal isolates were *A. fumigatus* and *F. oxysporum* (Figure 1A). On the other hand, the crude extracts of the thirty nontoxin producing fungal isolates that were tested showed no significant inhibition of *C. vulgaris* at the three time intervals used when compared with the control (Figure 1D, E and F). These results are in a agreement with those obtained by Bean et al. (1992) in which *C. vulgaris* proved to be the most sensitive organism to macrocyclic tichothecenes produced by *Myrothecium* species. Youssef et al. (2008) found that 14 out of 60 peanut seed samples that were tested inhibited the growth of *C. vulgaris.*

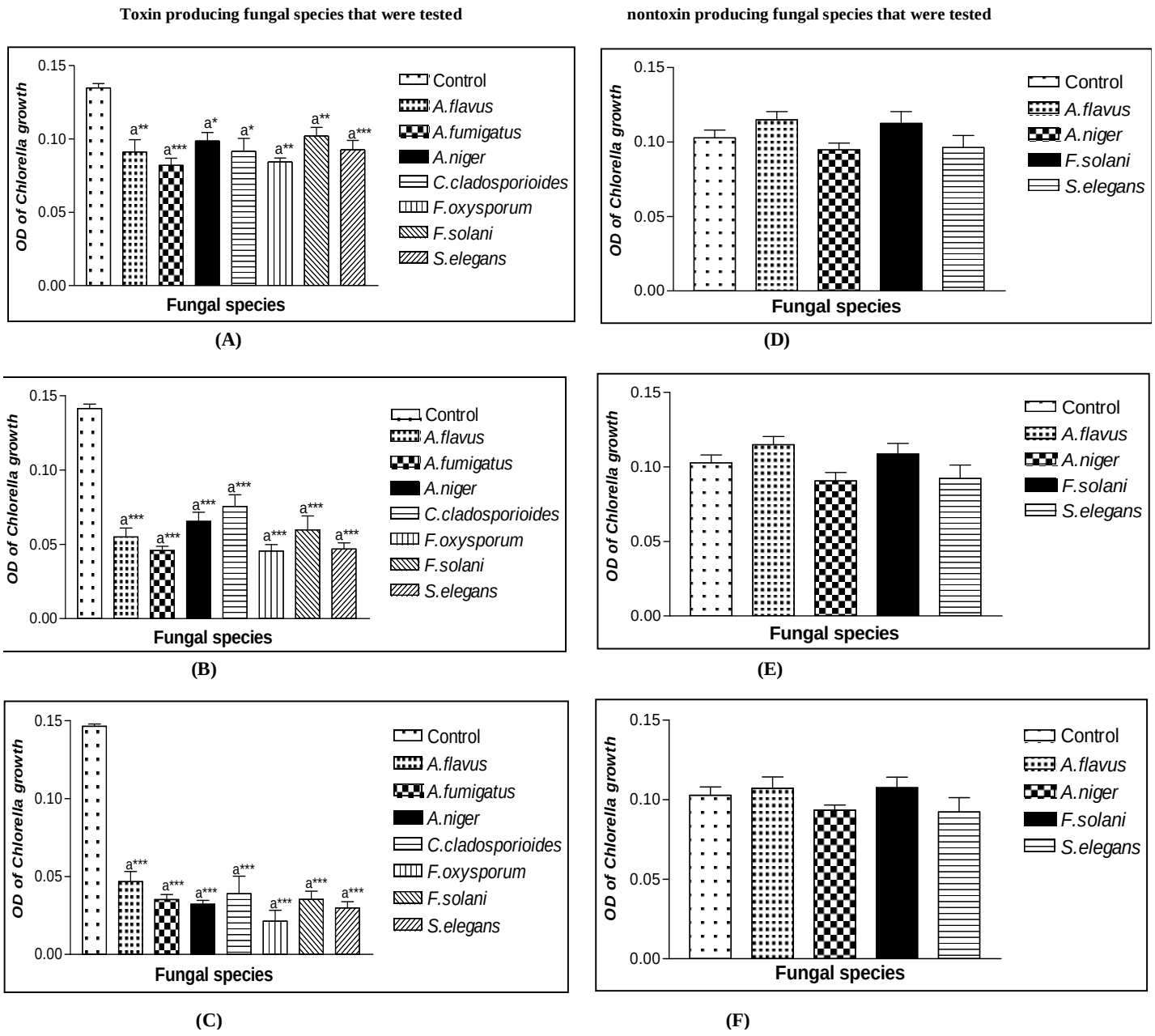

Figure 1. OD of *C. vulgaris* growth inhibited by the crude extracts of fungal isolates that were tested after 24 h (A and D), 48 h (B and E) and 72 h (C and F) of incubation. a= Significant difference between control group and fungal species. *= P< 0.05; ** = P< 0.01; *** = P< 0.001.

When the disk diffusion method (Sleigh and Timburg, 1981) was used to evaluate the antibacterial activity of the 78 fungal crude extracts of the toxic fungal, isolates on *B. subtilis* showed highly significant inhibition zone of which *S. elegans, A. fumigatus* and *A. flavus* were the most toxic isolates used (Figure 2). Several *Bacillus* species have been shown to be sensitive to other mycotoxins (Madhyastha et al., 1994). There was a great diversity in the response of the bacterial strains to fusaric acid; however, as a group, *Bacillus* species and *Paenibacillus macerans* were much more sensitive to fusaric acid than *Pseudomonas* species (Landa et al., 2002). Relative insensitivity of aflatoxin B towards *B. subtills* has been reported by Eka and Zo (1972). Reiss (1975) reported that the bioassay of patulin with *B. subtills* spores can be recommended as a sensitive technique to supplement TLC identification. In addition, Aboul-Nasr and Abdul-Rahman (2013) found that Gram positive bacteria (*Bacillus cereus* and *B. subtilis*) especially *B. subtilis* showed the highest sensitivity towards 76 *Fusarium* isolates producing FB_1 and FB_1 plus FB_2 tested.

The results of *C. vulgaris* were confirmed by those of

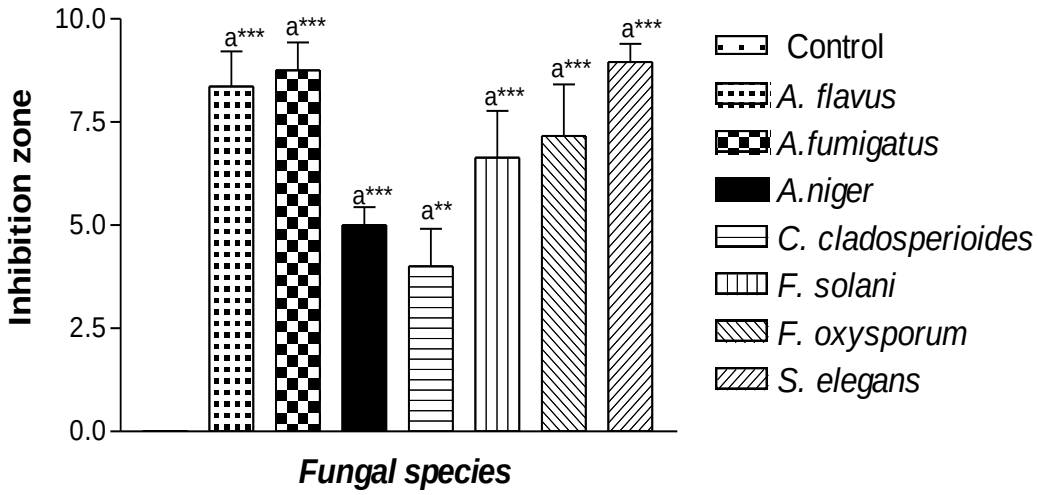

Figure 2. The inhibition zone of *B. subtilis* growth by toxin producing fungal isolates that were tested. A = Significant difference between control group and each fungal species group. *= P< 0.05; ** = P< 0.01; ***= P< 0.001.

bacteria by the proportional resemblance according to the method used either by the increase of inhibition zone in bacteria or the decrease of growth in *C. vulgaris.*

Conclusion

The results obtained by *C. vulgaris* procedure demonstrated a reliable short-term method for assessing the toxicity of mycotoxins and it can be performed easily.

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Full Length Research Paper

Characterisation of a *Bifidobacterium* sp. strain isolated from human faeces and its expression of the *ack* and *ldh* genes

Olvera Rangel N., Gutiérrez Nava A.*, Azaola Espinosa A. and Mayorga Reyes L.

Departamento de Sistemas Biológicos, Universidad Autónoma Metropolitana, UnidadXochimilco. D.F. C.P. 04960, México.

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Faecal samples of two Mexican breast-fed infants were used to isolate a *Bifidobacterium* strain in a selective medium. This strain was characterised biochemically for carbohydrate fermentation patterns and the fructose-6-phosphate phosphoketolase assay; genetically it was identified by PCR using genus-specific primers. The isolated strain was named *Bifidobacterium* JCLA3 and was grown on four different carbon sources; the respective growth kinetics of glucose, lactose, inulin and sucrose were measured. The highest cellular yield was presented in the culture media containing sucrose, followed by inulin, while on glucose and lactose, the cellular yield was lowest; these results correlate with the characteristic that bifidobacteria prefer complex carbon sources. We also determined the expression of the *ack* and *ldh* genes. The *ldh* gene presented high levels of expression regarding the *ack* gene; nevertheless on sucrose, the expression was lower regarding the other three substrates. The highest expression level of the *ack* gene was from the culture media with glucose, followed by inulin, sucrose and lactose.

Key words: *Bifidobacterium*, characterisation, gene expression.

INTRODUCTION

Bifidobacteria represent a significant proportion of the infant and adult gut microbiota. The beneficial effects of bifidobacteria on human health have been demonstrated in immunopotentiation, nutrition, prevention of intestinal infections, reduction of intestinal putrefaction as well as being used in functional foods and pharmaceutical products (Gibson and Roberfroid, 1995; Eckburg et al., 2005).

The isolation of bacteria from gut microbiota is important to characterise and analyse differences among strains. Selective and differential media have been described for the isolation of *Bifidobacterium* spp., nevertheless their identification is not clear because it is based on phenotypic characteristics that do not always provide reliable results. It is also known that bifidobacteria can change their morphology depending on the culture medium, growth and culture conditions (Bonaparte et al., 2001; Klein et al., 1998). Colonies have been identified as members of the genus *Bifidobacterium* by the detection of fructose-6-phosphate phosphoketolase (F6PPK) (Grill et al., 1995). However, the test is time consuming and laborious (Scardovi, 1986; Biavati et al., 1992; Tannock, 1999).

The use of 16S rRNA gene sequences to study bacterial phylogeny, taxonomy and genus and species identification has been the most common (Woese, 2000). 16S rRNA-targeted hybridization probes or PCR primers enable rapid and specific detection of a wide range of bacterial species, and have become key procedures in the

*Corresponding author. E-mail: agutz@correo.xoc.uam.mx.

detection of microorganisms (Kaufmann et al., 1997).

Like most intestinal bacteria, bifidobacteria are saccharolytic, they obtain carbon and energy through fermentation of host and dietary carbohydrates and show remarkable adaptations to use and metabolize complex oligosaccharides as carbon and energy sources (Lee and O'Sullivan, 2010).

Bifidobacteria are unable to carry out the usual glycollysis pathway or the hexose monophosphate shunt pathway; they possess only one metabolic pathway for the fermentation of hexoses- the bifidus shunt (De Vries et al., 1967). This pathway is dependent on the presence of fructose-6-phosphate phosphoketolase (F6PPK) (Fandi et al., 2001). Bifidobacteria catabolize a variety of mono- and oligosaccharides (Crociani et al., 1994) released by glycosyl hydrolases, with the formation of acetate, lactate, ethanol and even little amounts of succinate, as end products (Schell et al., 2002).

The main impact of these metabolites in human health is in the colon, decreasing the pH, which helps protect the intestine against pathogen colonization, and colon cancer by reducing the bioavailability of the toxic amines. The presence of acetate also helps the absorption of calcium and magnesium, improving blood flow in the colon and in the liver (Fukuda et al., 2012). Very little is known about the analysis of the expression of genes encoding the enzymes responsible for the production of the main metabolites of bifidobacteria.

The purpose of this study was to isolate a strain of the *Bifidobacterium* genus from human faeces and then characterise it biochemically and genetically, as well as analyse the levels of expression of the *ack* and *ldh* genes, coding for the acetate kinase (EC 2.7.2.1) and lactate dehydrogenase (EC 1.1.1.27) enzymes, respectively. The strain was tested in four different carbon sources.

MATERIALS AND METHODS

Isolation and bacterial identification

Faecal samples were obtained from two Mexican breast-fed infants, and explicit informed oral consent was obtained from their parents. Samples were homogenized in sterilized phosphate-buffered saline (PBS), and 1-mL aliquots were used to inoculate trypticase-phytone-yeast extract broth (TPY) supplemented with 1% Raftilose (Megafarma) (w/v). Samples were incubated at 37°C for 72 h under anaerobic conditions in an anaerobic system (Forma Scientific). Decimal dilutions were made in PBS and aliquots were plated on TPY agar supplemented with Raftilose. Colonies were identified as members of the genus *Bifidobacterium* by Gram staining; cellular morphology, carbohydrate fermentation patterns (API 50 CHL system, BioMerieux) and a fructuose-6-phosphate phosphoketolase (F6PPK) assay (Grill et al., 1995). The identity of a presumptive *Bifidobacterium* isolate was confirmed by genus-specific PCR (Kaufmann et al., 1997). The PCR product was subjected to agarose gel electrophoresis (1%) followed by GelRed stain (Biotium). The fragment was purified with the QIAquick Gel Extraction Kit (Qiagen) and sequenced.

Growth on different carbon sources

An inoculum was prepared in vials with 40 mL of trypticase-phytone-yeast extract broth (TPY) for each of the carbon sources: glucose, lactose, inulin and sucrose. The cultures were incubated in anaerobic conditions at 37°C for 8 h at 200 rpm in an Orbital Incubator Chamber (Gallenkamp). These cultures were used to inoculate fresh medium with their respective carbon sources at an initial optical density of 0.05. They were incubated under the same previous conditions, and samples were collected every 1.5 for 6 h (exponential growth rate). All the cells were harvested by centrifugation at 3,000 xg, washed twice in 0.1 N Tris-HCl pH 7.0 and stored in aliquots at -80°C for further analysis. All the fermentations were carried out in triplicate.

Survival and growth of *Bifidobacterium* JCLA3 at different pH conditions

To investigate the acid resistance of *Bifidobacterium* sp. JCLA3, previous TPY/glucose culture media with cells at pH 2, 3 and 4 were stored for 6 months at -20°C. Defrosted culture media were resuspended and centrifuged at 3,000 xg at 4°C for 5 min and washed twice in saline solution. Two milliliters of each cellular suspension was inoculated into vials with 40 mL of TPY/glucose medium at pH 2.0, 3.0 and 4.0, respectively. Cultures were incubated at 37°C and 200 rpm in anaerobic conditions. Samples were taken every 2 for 8 h of fermentation to determine the pH, optical density (OD) and cellular viability.

DNA and RNA isolation

Purified DNA of the isolated bacteria was obtained from a TPY/glucose culture, using the Wizard Genomic DNA Purification Kit (Promega). Samples of the strain growing on glucose, lactose, inulin and sucrose were collected in the exponential phase; RNA was isolated according to the protocol SV Total RNA Isolation System (Promega). The concentration of isolated DNA and RNA was determined using a NanoDrop spectrophotometer (Thermo Scientific, USA) and its quality was analysed by agarose gel electrophoresis (1%) with GelRed (Biotium).

Sequence analysis

The sequence of the purified fragment was determined by the dideoxy chain termination method, using the protocol provided in the Sequenase DNA sequencing kit (US Biochemical Corp.). To determine the close relatives of the partial 16S rDNA sequences, a search in the GenBank DNA database was conducted using the BLAST algorithm.

Expression of the *ack* and *ldh* genes by RT-PCR

For expression experiments, primers to amplify a 245-bp fragment of the *ack* gene were designed using the sequence reported for *Bifidobacterium longum* subsp. *infantis* ATCC 15697 (GenBankNC_017219), forward: 5'-CTCGGCTTCTTCGAGGAGTA-3', reverse: 5'-AAGGAGGAGTCGAACACGAA-3'. For the analysis of the *ldh* gene, two primers were designed to amplify a 208-bp fragment using the sequence of *Bifidobacterium infantis* (GenBank: FJ455841.1) forward: 5'-CACGGCTCCAGCTTCTACTC-3', reverse: 5'-TGAGCATGTAGATGGCGTTC-3'. As a housekeeping gene, a fragment of the constitutive gene xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (*xfp*) was used. Primers were designed from *B. longum* sequence (GenBank: AY377410), forward: 5'-CGGCCACGGCTGGGGCC-3', reverse: 5'-TCCTGACGCCAGACGTGGG-3'.

The expression levels of *ldh, ack* and *xfp* genes were analysed through the One Step Reverse Transcription kit (Qiagen) following the manufacturer's instructions. An amount of 0.3 µg of total RNA

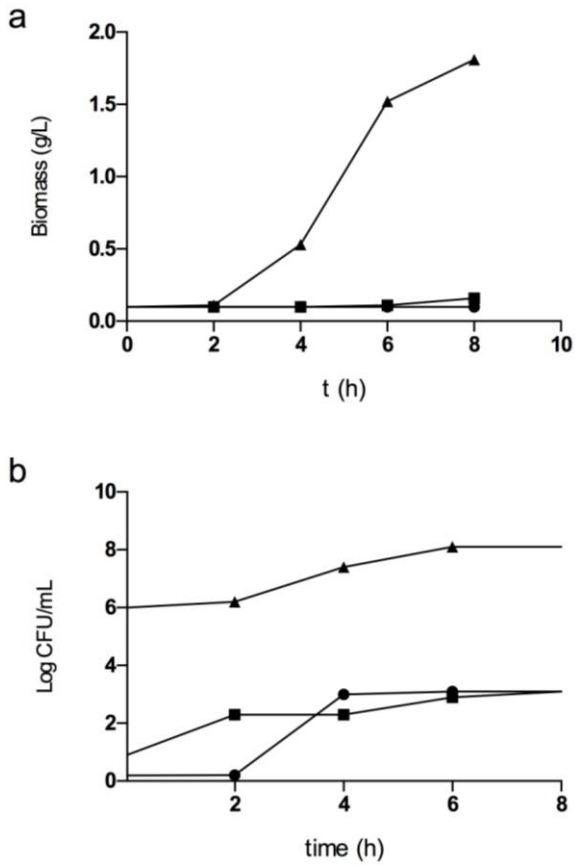

Figure 1. Kinetics of growth (1a) and viability (1b) of *Bifidobacterium* sp. JLCA3 stored for 6 months at -20°C, pH 2.0 (σ), pH 3.0 (ν) and pH 4.0 (●).

was used for each reaction. Reaction conditions were: with reverse transcriptase Superscript II at 50°C for 30 min, followed by specific amplification of the genes (5 min at 94°C, followed by 30 cycles at 94°C for 60 s, 54°C for 60 s and 72°C for 1 min). After amplification, RT-PCR products were analysed in 1.5% agarose gels stained with GelRed (Biotium) for luminescence in a UV transilluminator (Syngene).

Amplicons (cDNA bands) were analysed by densitometry with Kodak Digital Science 1D 3.6 software. Numeric values for the cDNA band intensities of the *Idh* and *ack* genes were normalized with the values of the *xfp* gene, as these express themselves at a relatively constant level in cells and are generally used in semi-quantitative systems of RT-PCR for analysing the relative efficiency of each individual PCR.

RESULTS

The isolated strain from breast-fed infant faeces was *Bifidobacterium* sp. strain JCLA3. This strain was F6PPK-positive and identified as *Bifidobacterium* which was confirmed by carbohydrate fermentation patterns using the API 50 CHL system and by PCR tools for molecular identification at the genus level. Nucleotide sequences were analysed using the BLAST tool. Approximately 30 sequences of different species of *Bifidobacterium* presented high similarity, as well as two sequences of *Gardnerella vaginalis,* which has a close phylogenetic relationship with the genus *Bifidobacterium* (Gavini et al., 1996). Based on the distinct phenotypic characteristics, Gram stain, biochemical and molecular genetics evidence, we confirm that the isolated strain belongs to the genus *Bifidobacterium.*

Growth kinetics

Comparative batch cultures of *Bifidobacterium* sp. JCLA3 on glucose, lactose, inulin or sucrose as sole carbon source were carried out. The strain was incapable of growing in TPY without the addition of carbohydrates. Increment of biomass was followed for 8 h identifying the exponential growth phase. The highest dry cell weight (DCW) production was found on sucrose (1.2 g/L) followed by inulin (0.32 g/L), while on glucose and lactose, the cellular production was 0.21 and 0.18 g/L, respectively. The cellular production was independent of the preference of each strain by the substrate; maximum specific rates for every culture were 0.492, 0.433, 0.275 and 0.274 h^{-1} on glucose, sucrose, lactose and inulin, respectively.

Survival and growth of *Bifidobacterium* sp. JCLA3 at different pH conditions

Bifidobacterium sp. JCLA3, stored at pH 2 at -20°C, maintained a viability of 10^6 to 10^8 CFU/mL after 6 months, whereas the viability of cells stored at pH 3.0 and 4.0 decreased 3 to 4 orders of magnitude. Each strain obtained at a different pH was inoculated on TPY/glucose medium; after 6 h of incubation, the cells at pH 2.0 increased to a cellular density of 1.8 g/L, whereas in the cultures of cells at pH 3.0 and 4.0, the growth was not detected (Figure 1a). The cell viability in the culture at pH 2.0 increased by 2 orders of magnitude after 6 h, unlike cells that were grown at pH 3.0 and 4.0 (Figure 1b), which did not show significant growth.

Expression of the genes *ack* and *Idh*

The expression of genes coding for acetate kinase (*ack*) and lactate dehydrogenase (*Idh*) enzymes were analysed by RT-PCR (Figure 2A and 2B, respectively). The RNA was obtained from the isolated bacteria *Bifidobacterium* sp. JCLA3 during its exponential growth phase on each of the four carbon sources. Comparing the expression levels of the *ack* gene, the highest expression appeared in glucose (42%), followed by inulin (33%), sucrose (29%) and lactose (13%). For the *Idh* gene, the expression levels were high in all cases, similar to the control gene: glucose (97%), followed by lactose and inulin (96%), and sucrose (71%) (Figure 2C). These numeric values for band intensity of cDNA were corrected with the values of the housekeeping *xfp* gene in order to analyse the relative expression of each gene (Figure 2C). With this internal control, it can be demonstrated that the same amount of RNA was used in every experiment, and differences in the expres-

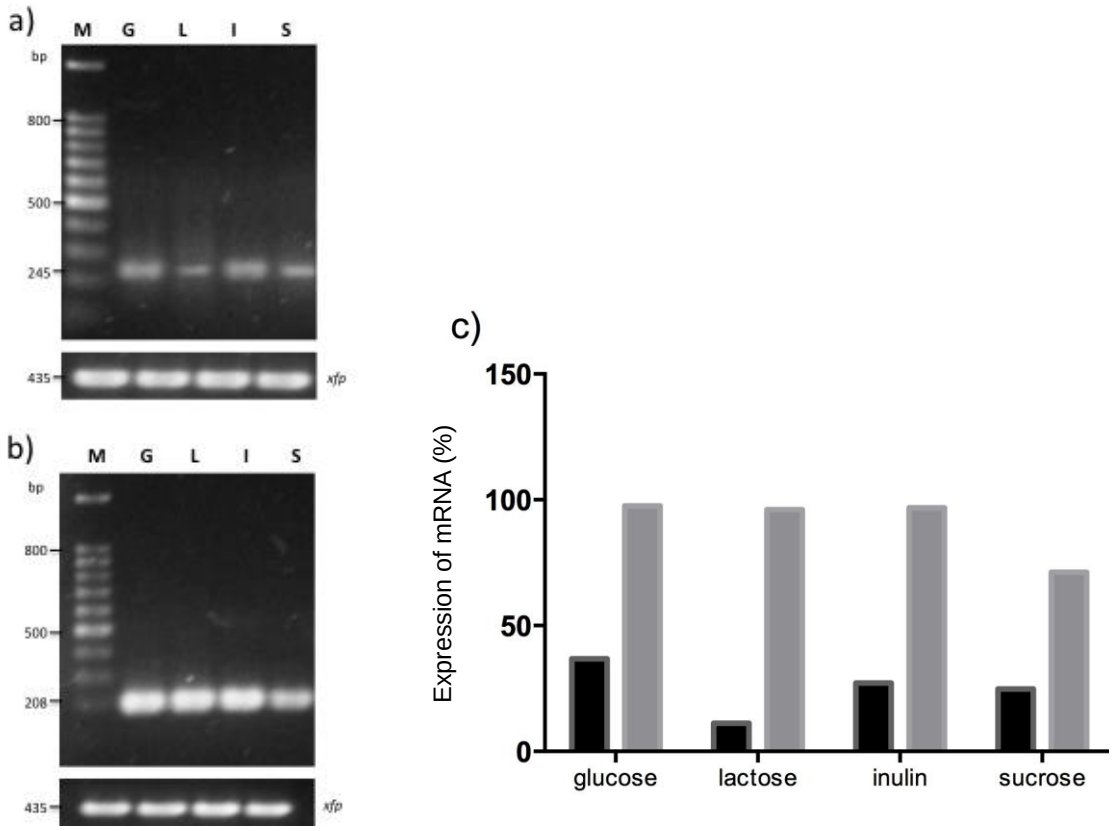

Figure 2. Expression of the *ack* (■) and *ldh* (■) genes by RT-PCR during exponential growth of the *Bifidobacterium* strain isolated from human faeces on G: glucose, L: lactose, I: inulin, and S: sucrose as carbon sources. Amplicons from 300 ng of RNA of the 245-bp *ack* (a) and 208-bp *ldh* fragments (b). Agarose gels of 1.5% stained with GelRed®. M: 100-bp DNA ladder. (c) Graphics representing percentage expression analyses by densitometry; the *xfp* housekeeping gene was used to normalize the data.

sion levels of *ack* were caused by the source of carbon used by *Bifidobacterium* sp. JCLA3.

DISCUSSION

Strain isolation of the genus *Bifidobacterium* from biological samples of the gastrointestinal tract, faeces and dairy products; have been carried out successfully in selective media developed over several decades. However, in order to differentiate them from other morphologically similar bacteria, such as *Lactobacillus, Actinomyces, Propionibacterium and Eubacterium,* biochemical identification by carbohydrate fermentation profiles and the presence of the fructose-6-phosphate phosphoketolase enzyme is important. Nowadays, the use of molecular tools for the detection, differentiation and identification of bifidobacteria has allowed surpassing the limitations that isolation from culture media involved. The design of genus-specific primers based upon the sequence of the 16S rRNA gene is the main analysis conducted in order to identify this microorganism (Kaufmann et al., 1997).

BLAST analysis of the 1.35-kbp fragment sequence amplified by PCR using these primers has allowed us to confirm that the isolated microorganism belongs to the genus *Bifidobacterium*. Nevertheless, the two sequences of *G. vaginalis,* which also showed high similarity cannot be considered due to the fact that this bacteria belongs to the *Bifidobacteriaceae* family, but they are Gram-negative bacilli.

The highest cell production of *Bifidobacterium* sp. JCLA3 was obtained in the sucrose and inulin media, while in glucose and lactose the cell production was low. Previous reports with mono- and oligosaccharides using different bifidobacteria strains have reported wide differences regarding the preference and consumption of oligosaccharides and their monomeric components. *Bifidobacterium lactis* grew better in raffinose than in lactose, and *Bifidobacterium longum* used preferably lactose over glucose when provided both as a carbon source (Trindade et al., 2003). Perrin et al. (2001) observed that biomass production of *B. infantis* ATCC 15697 was similar in sucrose and glucose; while Amaretti et al. (2007) observed that the cellular yield of *Bifidobacterium adolescentis* MB 239 was highest when growing in galactose, followed by lactose and GOS, and the lowest in the presence of glucose.

The growth rate of *Bifidobacterium* sp. JCLA3 was highest when using glucose, followed by sucrose, lactose and inulin. However, the adaptation phase to the glucose medium was higher, followed by sucrose, lactose and inulin. The adaptation phase for glucose was 3 h more than the other three substrates. Specific growth rates reported for other bifidobacteria strains are better in complex substrates than in their monomeric units (Gibson and Wang, 1994; Hopkins et al., 1998; Rada et al., 2002; Kim et al., 2003; Palframan et al., 2003; Van der Meulen et al., 2004; Amaretti et al., 2006, 2007).

A wide variety of results have been observed for the utilization of carbon sources by bifidobacteria. In some cases, monosaccharides are mainly used over oligosaccharides (Mlobeli et al., 1998; Van der Meulen et al., 2006); whereas in other species, cellular performances are better in disaccharides and oligosaccharides than in monosaccharides (Kim et al., 2003; Amaretti et al., 2006; Hopkins et al., 1998). *B. lactis* grew better in raffinose than in lactose, and *B. longum* used lactose preferably over glucose when both were available as carbon sources (Trindade et al., 2003). These results can be directly related to the transport systems and their major efficiency for oligosaccharides than for the monomeric components (Trojanova et al., 2006).

The fermentation of prebiotics by bifidobacteria may promote some specific physiological functions through the release of metabolites from the bacteria, especially short chain fatty acids (acetate, propionate and butyrate) and organic acids (lactic acid) into the lumen of the intestine. Short chain fatty acids (SCFAs) may act directly or indirectly (by modifying the pH) on intestinal cells and may be involved in the control of various processes such as the proliferation of mucosa, inflammation, colorectal, carcinogenesis, mineral absorption and the elimination of nitrogenated compounds. Also, SCFAs stimulate sodium and water adsorption in the colon and are known for their ability to induce enzymes that promote mucosal restitution (D'Argenio and Mazzacca, 1999). In addition, lactic acid has an inhibitory effect on *Salmonella enterica* (Makras et al., 2006).

The ability of *bifidobacterium* to survive in acidic conditions depends on the species and source. Matzumoto et al. (2004) reported the viability of *B. longum* and *B. adolescentis* at pH 3.0. Charteris et al. (1998) observed a resistance to acidic conditions for 90 min in *B. bifidum, Bifidobacterium animalis, Bifidobacterium breve and B. infantis*. Probiotic bifidobacteria decreased in numbers very slowly in frozen storage, but are known to die more rapidly at refrigeration temperatures (4°C) (Maus and Ingham, 2003), possibly as a result of sensitivity to oxygen (Shimamura et al., 1992).

Bifidobacterium bifidum was found to be tolerant to the acidity of a gastrointestinal tract system model, with only a 20% decrease in numbers as the pH decreased from 5.0 to 1.8 over an 80-min period (Martteau et al., 1997). Generally, *Bifidobacterium* have been subjected to sublethal acid stress conditions and little has been reported on prolonged acid exposure. When the bacteria were expo-sed to acidic conditions, the homeostatic pH was main-tained by releasing the cell H^+. The F_1F_0-ATPase proton pump was responsible for the survival of some organisms in acidic environments (Cotter and Hill, 2003). Furthermore, this system was dependent on the activity of the ATPase enzyme responsible for maintaining the concentration of H^+ between the cell and environment (Matzumoto et al., 2004; Saarela et al., 2004).

The expression levels of both genes (*ack* and *ldh*) were higher while growing on glucose and inulin, suggesting that production of acetate and lactate were favored in these carbon sources. The reason for the low expression levels of the *ack* gene may be because in the bifidus shunt, acetate is produced in two points of the pathway (Suzuki et al., 2010) and the acetate kinase enzymes could be different; Genbank reports at least two different sequences for acetate kinases in the genus *Bifidobacterium*.

In previous studies with bifidobacteria, it has been demonstrated that *B. longum* NCC2705 growing on MRS medium with different monosaccharides as carbon sources, has its major production of acetic acid using glucose, nevertheless, this production did not measure up in other more complex sources of carbon (Liu et al., 2011). Comparative studies of the growth of *B. adolescentis* MB 239 in SM medium with glucose, galactose, lactose and GOS as carbon sources, showed a major production of acetic acid in GOS (280.6 mM) regarding other substrates: 182.3, 218.6, 202.8 mM, respectively (Amaretti et al., 2007). The inhibitory effects of bifidobacteria against various pathogens including *E. coli, Shigella dysenteriae* and *Yersinia enterocolitica* were demonstrated by numerous *in vitro* studies, and the mechanism of inhibition could be related to the production of acetic and lactic acids (Biavati et al., 2000).

Conclusions

The isolation of an acid-resistant *Bifidobacterium* strain from human faeces is important to know the microbiota of a given population. The strain could be used as a probiotic to improve human health by protecting the host from enteropathogenic infections by the high levels of *ack* and *ldh* expression. The production of acetic and lactic acids as the main metabolites of the bifidus shunt improves intestinal defence mediated by epithelial cells and, thereby, protects the host against lethal infection. It has been demonstrated at the molecular level that acetate inhibits the translocation of a toxin of *E. coli* from the gut lumen to the blood and that lactic acid has an inhibitory effect over other enteropathogenic strains.

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Effect of salting and packaging on liquid-smoked rainbow trout fillets during refrigerated storage

Pinar OĞUZHAN

Food Engineering Department, Faculty of Engineering, Ardahan University, TR-75000 Ardahan, Turkey.

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The aim of this study was to investigate the effect of salting and packaging treatments on liquid-smoked rainbow trout fillets during refrigerated storage (4°C) for 120 days. Treatments included the following: V1 (vacuum packaged- 35% brine), M1 (modified atmosphere packaged- 50% CO_2 + 50% N_2- 35% brine), V2 (vacuum packaged- 70% brine) and M2 (modified atmosphere packaged- 70% brine). Fillets were subjected to microbiological (total aerobic mesophilic bacteria, psychrotrophic bacteria, *Pseudomonas,* lactic acid bacteria, Enterobacteriaceae, yeast and mould) and chemical (pH, thiobarbituric acid reactive substances-TBARS, total volatile base nitrogen-TVB-N) analyses on 0, 15, 30, 45, 60, 75, 90, 105 and 120th days. The number of microorganisms, TBARS and TVB-N values increased according to increment of storage. Modified atmosphere packaging (MAP) gave better results than vacuum packaging in terms of all investigated parameters. Moreover, M2 was effective in extending the shelf life of rainbow trout fillets.

Key words: *Oncorhynchus mykiss,* liquid smoking, modified atmosphere packaging (MAP), vacuum, brine salting, shelf life.

INTRODUCTION

Microorganisms can reproduce in the muscle tissue of aquatic products because these are not only sensitive but also have rich nutrient content. So, the conservation and handling of aquatic products are very important (Babadoğan, 1998). One of the conservation processes of aquatic products is smoking (Bellagha et al., 2007). Smoking is one of the oldest methods of food presser-vation and widely used in fish processing (Muratore and Licciardella, 2005; Stolyhwo and Sikorski, 2005).

The shelf-life of smoked fish products depends largely on the intial bacterial contamination of the raw material; on the decrease in the water activity (aw) of the tissues due to brining and pre-drying; on the activation of putrefactive microflora due to the heat treatment; on the amount of smoke compenents that penetrate the product and on the temperature, air humidity and oxygen levels during storage (Sikorski et al.,1990; Ibrahim et al., 2008).

There are three methods used to smoke fish: the traditional method by combusition, at either low temperature (cold smoking ≤30°C) or high temperature (hot smoking ≥60°C); use of a high voltage electrostatic field which accelerates smoke deposition; and use of liquid smoked fish (Goulas and Kontominas, 2005). Liquid smoke flavorant is obtained from condensation of wood smoke and generally used as a flavoring agent (Siskos et al., 2005). Easy application, speed, product uniformity, low price and compatibility with the environ-ment are the advantages of the use of smoke flavorings

E-mail: pinaroguzhan@ardahan.edu.tr. Tel: +90 478 2115000. Fax: +90 478 2113275.

as compared to traditional smoking techniques (Simon et al., 2005; Alçiçek, 2011). Salting process is itself a preservation technique, is used as a preliminary operation for many processing technology (smoking, drying and marinating processes). The first step of the fish smoking process is salting. The main purpose of salting the fish meat is a part of the elimination of water. Bacterial activity is largely prevented in high salt concentrations. High salt concentration prevents bacterial activity which can generate spoilage in fish. Consequently, salting process, significantly increase the shelf-life of fish (Ismail and Wootton, 1992).

Beyong the prevention of microbial growth and reduction of water activity, NaCl is an essential ingredient in processed meat products for its contribution to the water-holding capacity, facilitating the solubilisation of certain proteins and conferring a typical salty taste by enhancing the flavour of such food products (Armenteros et al., 2009). Vacuum packaging method is a type of passive modified atmosphere. After placing the food in a suitable packaging material in this operation, the air in the package is emptied by vacuum and a hermetic closure (air tight) is made. This method is a rarely used for preservation of meat products. Even in vacuum packaging a low percentage of O_2 remain in the package and this can be used by aerobic microorganisms; with CO_2 production. In these types of products, the bacterial growth and oxidation of the product is prevented as air is not in the package (Keleş, 1998; Gülyavuz and Ünlusayin, 1999; Kilinç and Çakli, 2001). Thus, numerous studies have been conducted on the preservation of vacuum on fish and fish products of salmon (Leroi et al., 2000; González-Rodríguez et al., 2002; Martinez et al., 2010), ascidia (Stamatis et al., 2008), sardine (Senesi et al., 1980; Gómez-Estaca et al., 2010) and trout (Schulze 1985; Lyhs et al., 1998; Frangos et al., 2010; Oğuzhan and Angiş, 2012).

Modified atmosphere packaging (MAP), is a preservation method use to extent shelf-life of fish and fish products (Özoğul et al., 2006). Modified atmosphere packaging, elimination of oxygen from inside package and filled with different concentrations of CO_2 and N_2, however, refrigerated storage conditions for aerobic microorganisms, proteolytic bacteria, yeast and mold growth is inhibited (Swiderski et al., 1997; Gülyavuz and Ünlüsayin, 1999; Kilinç and Çakli, 2004). There are many studies of MAP related to shelf life extension of fish and fishery products of swordfish (Stolyhwo and Sikorski, 2005), chub mackarel (Erkan and Özden, 2007), cod (Cann, 1983; Debevere and Boskou, 1996), rainbow trout (Çakli et al., 2006; Oğuzhan and Angiş, 2012), salmon (Paludan-Müller et al., 1998), bass (Torrierri et al., 2006), herring (Lyhs et al., 2007) and catfish (Göktepe and Moody, 1998).

The aim of this study was to determine the effects of salting and packaging (vacuum and MAP) on the shelf-life of refrigerated (4°C) liquid smoking rainbow trout fillets by evaluating microbiological and chemical parameters.

MATERIALS AND METHODS

Preparing samples

Rainbow trout (*Oncorhynchus mykiss*) (250±25 g) were obtained from Rainbow Trout Breeding and Research Center, Fisheries Department, Agricultural College, Ataturk University. Fish were carried to laboratory and washed with tap water. A total of 72 fish samples were eviscerated, stored until rigor had resolved and then filleted, 144 fillets in total (Robb et al., 2002).

Fillets were divided into fours groups (36 samples each) according to brine salting process and packaging treatment: V1 (vacuum packaged-35% brine), M1 (modified atmosphere packaged 50% CO_2+50% N_2-35% brine), V2 (vacuum packaged-70% brine) and M2 (modified atmosphere packaged 50% CO_2 + 50% N_2-70% brine).

Brine salting process

Fillets were washed to remove blood and mucous remains and were divided into two groups. The first and second groups were submitted to 35 and 70% brine salting (NaCl) at 4°C for 4 h, respectively. Liquid smoke flavouring (GMT Food Ingredients CO., Istanbul-Turkey) (100 ml in 1 L of brine solutions) was added into two groups and fillets were dried for 30 min. After drying, samples were smoked at 80-90°C. Fillets were packed by applying vacuum and modified atmosphere (50% CO_2+50% N_2).

Vacuum and modified atmosphere packaging

All filleted samples including the control were packaged and obtained from the firm Südpack Verpackungen GmbH+Co (Germany) 15 x 25 cm PA/PE (Polyethylene/Polyamide) (3-seal bags GB 70) in thickness having an O_2 permeability of 40 cm^3/m^2/day.atm.23°C; N_2 permeability of 24 cm^3/m^2/day.atm.23°C; CO_2 permeability of 145 cm^3/m^2/day.atm.23°C and a water vapour permeability of <3 g/m^2/day.atm.23°C).

After brine salting process and packaging treatment rainbow trout fillets were stored under refrigeration (4±1°C) and were subjected to microbiological (total aerobic mesophilic bacteria, psychrotrophic bacteria, *Pseudomonas*, lactic acid bacteria, Enterobacteriaceae, yeast and mould) and chemical (pH, thiobarbituric acid reactive substances-TBARS, total volatile base nitrogen- TVB-N) analyses. Microbiological and chemical procedures were performed on 0, 15, 30, 45, 60, 75, 90, 105 and 120th days of storage.

Microbiological analysis

A sample (25 g) was taken from each group, transferred aseptically into a stomacher bag containing 225 ml of 0.1% peptone water and was homogenized for 60 s in Stomacher (Lab Stomacher Blender 400-BA 7021 Sewardmedical, England) at room temperature. For microbial analyses, 0.1 ml samples of serial dilutions (1:10, diluent, 0.1% peptone water) were inoculated onto proper agar plates. Total mesophilic aerobic bacteria (TMAB) and total psychrotrophic aerobic bacteria (TPAB) were determined on Plate Count Agar (PCA, Merck 1.05463.0500), which were incubated at 30°C for two days and at 10°C for 7 days, respectively. *Pseudomonads* were

determined using cetrimide fusidin cephaloridine agar (CFC, Pseudomonas Agar Base-Oxoid CM0559 + CFC Selective Agar Supplement-Oxoid SR0103) after incubation at 25°C for 2 days. Lactic acid bacteria (LAB) were determined by using Man Rogosa Sharpe agar (MRS, de Man, Rogosa Sharpe Agar Oxoid CM0361), which was incubated at 30°C for 2 days. Enterobacteriaceae count were performed (Violet Red Bile Dextrose Agar Merck 1.10275.0500) which was incubated at 30°C for 2 days. Yeast and mould were determined on Rose Bengal Chloramphenicol (RBC) Agar (Merck 1.00467.0500), which was incubated at 25°C for 5 days.

Chemical analysis

Total volatile base nitrogen (TVB-N) was determined according to Malle and Tao (1987). TVB-N contents were expressed as mg 100/g fish muscle. Thiobarbituric acid reactive substances (TBARS) was determined according to the method of Lemon (1975) and Kiliç and Richards (2003). TBARS content was expressed as µmol malondialdehyde (MDA)/kg fish muscle. pH was determined according to the method of Gökalp et al. (1999).

Statistical analysis

Experiments were replicated twice on two separate occasions with different fish samples. Analyses were run in duplicate for each replicate. All obtained data from this study were subjected to analysis of variance (ANOVA), and followed by Duncan's multiple range test to determine significant differences among means at $\alpha = 0.05$ level by using SPSS (1999).

RESULTS

Microbiological changes

Changes in TMAB of refrigerated rainbow trout fillets during storage under vacuum and modified atmosphere packaging are shown in Figure 1a. The initial count (day 0) of TMAB was 2.0 log cfu/g. V1, M1 and V2 exceeded the limit (7 log cfu/g) for fresh marine species (ICMSF, 1992) on days 90, 105 and 105 of storage, respectively. This limit was not exceeded throughout storage in M2. At 120 days, V1, M1, V2 and M2 showed 9.68, 8.29, 8.05 and 5.45 log cfu/g respectively.

The initial (day 0) psychrotrophic bacteria (Figure 1b) of rainbow trout fillets was 2.0 log cfu/g. V1, M1 and V2 exceeded the value of 7 log cfu/g for psychrotrophic bacteria, which was considered as the upper acceptability limit for fresh marine species (ICMSF, 1992) on days 90, 105 and 105 of storage, respectively. This limit was not exceeded throughout storage in M2. The counts of V1, M1, V2 and M2 were 9.65, 8.22, 8.24 and 5.83 log cfu/g at the end of storage, respectively.

Pseudomonas (Figure 1c) show inital count (day 0) of 2.0 log cfu/g. At the end of storage, V1, M1, V2 and M2 reached 7.02, 5.96, 5.02 and 3.50 log cfu/g, respectively. Initial counts were 2.0 log cfu/g (LAB) (Figure 1d) and 2.0 log cfu/g (Enterobacteriaceae) (Figure 1e). At the end of storage, values of LAB (7.99, 6.34, 5.50 and 4.40 log cfu/g) and Enterobacteriaceae (6.42, 5.39, 5.01 and 3.46 log cfu/g) were recorded for treatments V1, M1, V2 and M2, respectively.

The yeast and mould count (Figure 1f) was 2.0 log cfu/g at 0 day. At the end of storage ,V1, M1, V2 and M2 showed 6.80, 5.80, 4.25 and 4.0 log cfu/g, respectively.

Chemical changes

TVB-nitrogen

The amount of TVB-N is an important criteriation in determining freshness of fish and their products because generally TVB-N values increase according to progression of spoilage process (Köse and Koral, 2005). TVB-N consist of TMA and ammonia with the effect of the bacteria and endogenous enzymes in fish. TVB-N value increase depend on the storage period in preservation of fish and fish products (Lannelongue, 1980; Öksüztepe et al., 2010). TVB-N values (Figure 2a) at 0 day were 18.12, 17.54, 17.99 and 16.81 mg N/100 g for V1, V2, M1 and M2, respectively. V1, M1 and M2 exceeded the limit (25 mg/100 g) for rainbow trout (Robb et al., 2002) on days 90 of storage, whereas M1 exceeded the value of 25 mg/100g on days 75 of storage.

Lipid oxidation

Lipid oxidation is one of the factor causing spoilage in product. Ransidity taste and yellow colour is seen in oxidation products (Ruiz-Capillas and Moral, 2001). Lipid oxidation is a major quality problem especially in fatty marine species. It leads to the development of off-odors and offtastes in edible oils and fat containing foods, known as oxidative rancidity. The TBARS value is an index of lipid oxidation measuring malondialdehyde (MDA) content. MDA is formed through hydroperoxides, which are the initial reaction products of polyunsaturated fatty acids with oxygen (Fernandez et al., 1998; Rezai et al., 2008). Initial TBARS values (Figure 2b) were 2.16, 2.06, 1.75 and 1.59 µmol malondialdehyde (MDA)/kg for V1, V2, M1 and M2, respectively. At the end of storage, TBARS values were 10.45, 7.22, 8.36 and 6.39 µmol malondialdehyde (MDA)/kg for treatments V1, M1, V2 and M2, respectively.

pH

pH value of fish meat usually ranges from 5.7-6.6. Fresh fish is close to neutral pH, after death, lactic acid will be formed firstly; falling and then rising again with spoilage (Bilgin et al., 2007). The pH values of rainbow trout fillets

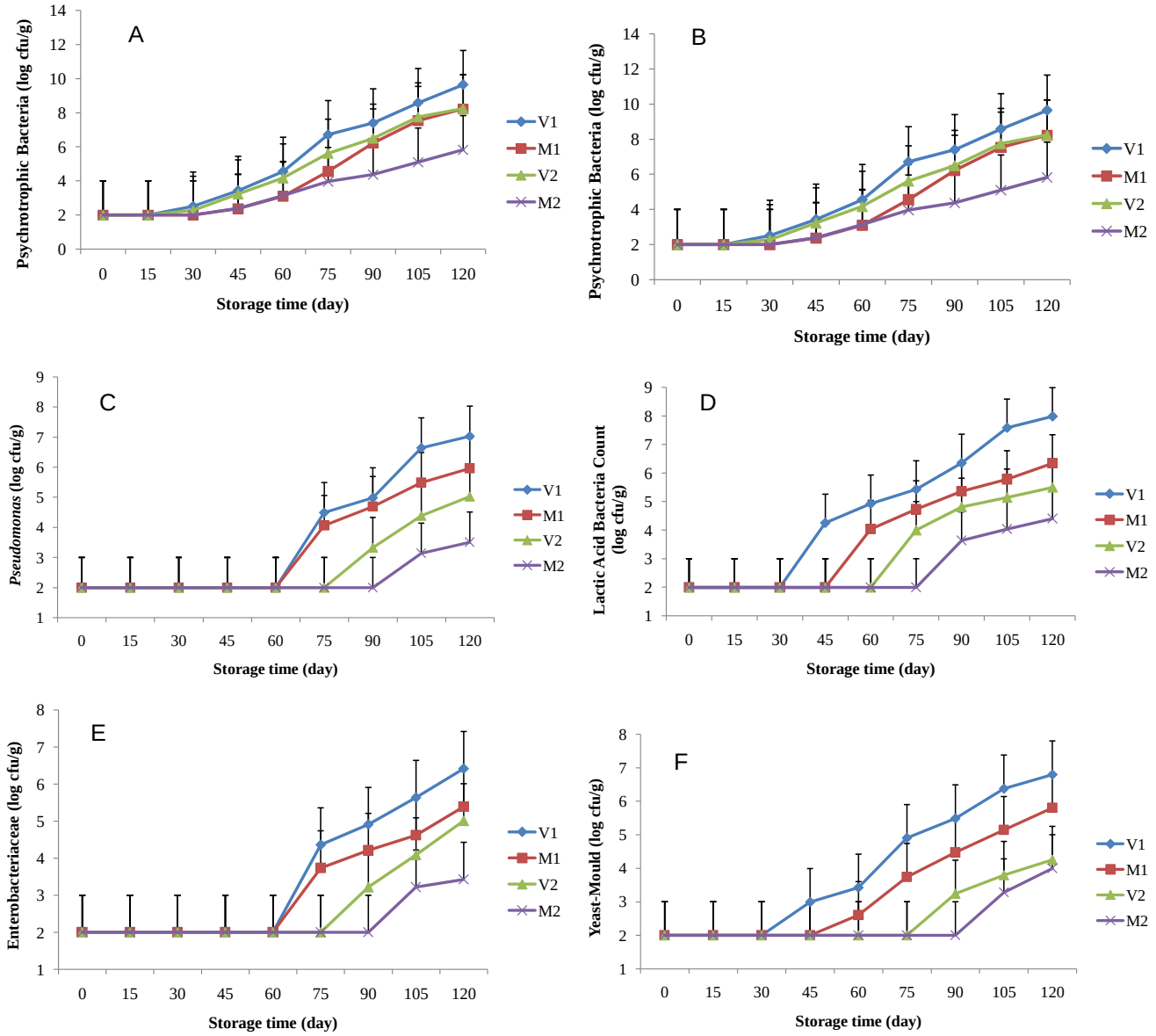

Figure 1. Total aerobic mesophilic bacteria counts (a), psychrotrophic bacteria (b), *Pseudomonas* (c), lactic acid bacteria counts (d), Enterobacteriaceae counts (e) and yeast and mould (f) changes of brined (35% and 70%) liquid smoking rainbow trout fillets during cold storage in vacuum and MAP conditions at 4°C.

(Figure 2c) were 6.15, 6.11, 6.15 and 6.02 (at initial experiment) and 6.5, 6.48, 6.42 and 6.41 (at end of storage) for V1, V2, M1 and M2, respectively.

DISCUSSION

Similar findings were reported by other researches (Dodds et al., 1992; Göktepe and Moody, 1998; Hansen et al., 1998; Kolsarici and Özkaya, 1998; Leroi et al., 1998; Paludan-Müller et al., 1998; González-Rodríguez et al., 2002; Dondero et al., 2004; Çakli et al., 2006; Erkan, 2012). Bacterial growth of modified atmosphere packaged samples was lower than vacuum packaged samples, probably, due to the presence of CO_2 gas in MAP. The carbon dioxide can be considered effectively inhibitory on the total mesophilic and psychrotrophic aerobic bacteria. Similar results were observed by several researchers (Leroi et al., 1998; Dondero et al., 2004; Çakli et al., 2006; Gümüş et al., 2008; Kilinç et al., 2009; Erkan, 2012; Frangos et al., 2010; Prygotou et al., 2010; Can, 2011; Oğuzhan and Angiş, 2012).

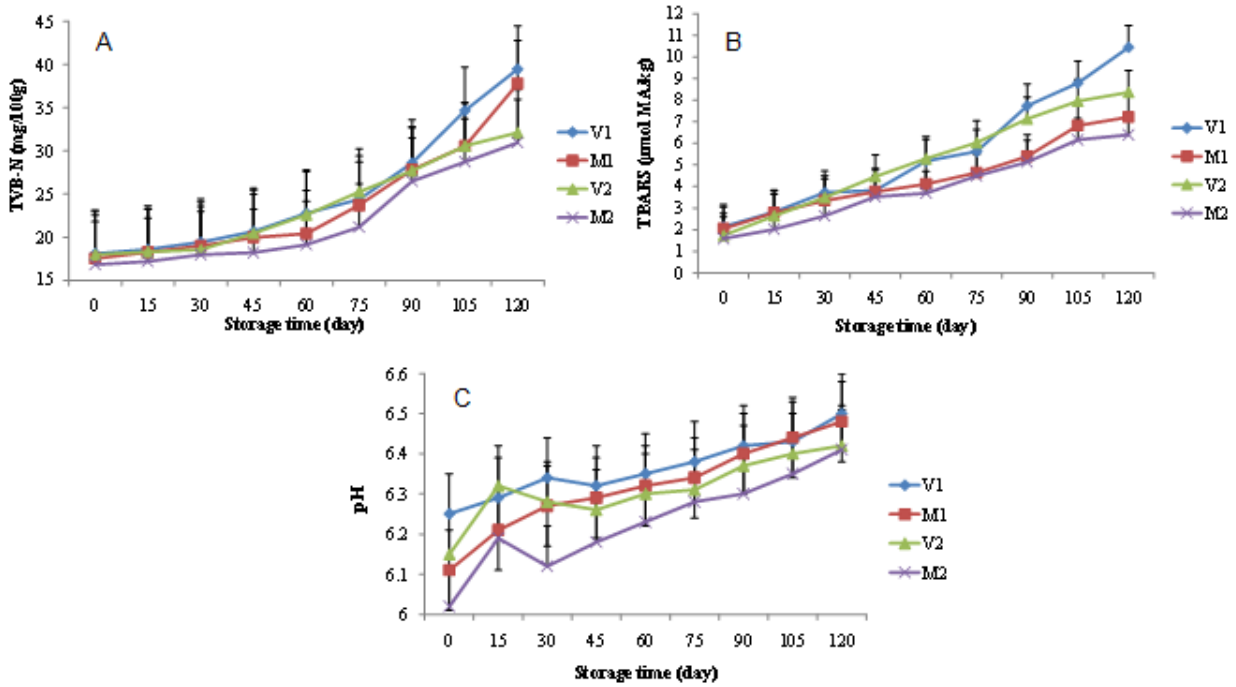

Figure 2. TVB-N (a), TBARS (b), pH (c) changes of brined (35% and 70%) liquid smoking rainbow trout fillets during cold storage in vacuum and MAP conditions at 4°C.

Pseudomonas spp. were recognised early as putative spoilage inducers in fish muscle and have since then been found in various fish species from fresh and marine waters as well as in other foods (Castell and Greenough, 1957; Macdonell and Colwell, 1985; Reynisson et al., 2009). This result is due to liquid smoking. *Pseudomonas* was seen in the duration of storage in all groups. Similar findings were observed by Jay (1986) for hot smoked roach and whitefish.

LAB are facultative anaerobic bacteria that can grow under both anaerobic and aerobic conditions (Plahar et al., 1991). The initial LAB numbers 2.0 log cfu/g while this values increased during storage time in all groups. Similar results were found for hot smoking rainbow trout (Kolsarici and Özkaya, 1998; Çakli et al., 2006).

Enterobacteriaceae, a hygiene indicator, was also part of the microflora of fresh rainbow trout. Similar findings were observed for cold smoking salmon by Hansen et al. (1998). Similar yeast and mould (day 0) were reported for herring by Plahar et al. (1991) and Kaya et al. (2006).

TVB-N may be considered as a quality index for fish. Its increase is related to the activity of bacteral spoilage and endogenous enzymes (Dodds et al., 1992; Göktepe and Moody, 1998; Erkan et al., 2007). According to quality classification, "very good" TVB-N value should be up to 25 mg/100 g, "good" up to 30 mg/100 g, "marketable" up to 35 mg/100 g and "spoilaged" more than 35 mg/100 g which were evaluated (Varlik et al., 1993). TVB-N values were 18.12, 17.54, 17.99 and 16. mg/100 g for V1, M1, V2 and M2 at initial experiment (day 0), respectively. This values increased in the duration of storage time in all groups. Similarly, TVB-N values have been reported for herring (Plahar et al., 1991), rainbow trout (Ünal, 1995; Çakli et al., 2006; Alçiçek, 2011), atlantic bonito (Duyar et al., 2008), mullet (İbrahim et al., 2008), mirror carp (Duman and Patir, 2007), sardine and dolphinfish (Gómez-Estaca et al., 2010).

TBA values represent the degree of the rancidity in the products and fresh fish is much lower than the acceptable upper limits of 8 mg malonaldehyde/kg (Schormüller et al., 1969). In "perfect material", TBA value should be less than 3 mg malonaldehyde/kg, in " good material" TBA value should not be more than 5 mg malonaldehyde/kg and consumption limit for TBA value is between 7 and 8 mg malonaldehyde/kg.

TBA values showed the degree of rancidity in the products, and values greater than 3-4 mg malonaldehyde/kg indicated a loss of product quality (Frangos et al., 2010). This values increased in the duration of storage time in all groups. Similarly, TBARS values have been reported for mirror carp (Duman an Patir, 2007), mullet (İbrahim et al., 2008), atlantic bonito (Koral et al., 2010) and salmon (Martinez et al., 2010).

The pH in fresh fish flesh is almost neutral. In the post-mortem period, decomposition of nitrogenous compounds leads to an increase in pH in the fish flesh. The increase

in pH indicates the loss of quality (Can, 2011). Similarly, pH values have been reported for chub mackerel (Goulas and Kontominas, 2005; Chatzikyriakidou and Katsanidis, 2012), salmon (Martinez et al., 2010) and rainbow trout (Alçiçek, 2011). pH values of modified atmosphere packaged group were lower than that of vacuum packaged samples. This result is due to carbonic acid conversion of carbon dioxide.

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Full Length Research Paper

Detection of *Yersinia pestis* DNA in human bubo aspirates in Tanzania

Ziwa M. H.[1,*], Hang'ombe B. M.[2], Lyamuya E. F.[1], Kilonzo B. S.[3], Simulundu E.[2] and Matee M. I.[1]

[1]Department of Microbiology and Immunology, School of Medicine, Muhimbili University of Health and Allied Sciences, P. O. Box 65001 Dar es Salaam, Tanzania.
[2]Microbiology Unit, Department of Paraclinical Studies, School of Veterinary Medicine, University of Zambia, P. O. Box 32379, Lusaka, Zambia.
[3]Pest Management Centre, Sokoine University of Agriculture, P. O. Box 3110, Morogoro, Tanzania.

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The use of molecular techniques to detect *Yersinia pestis* has enabled remarkable progress in the provision of necessary information on the occurrence of plague. In Tanzania, despite the long history of plague, DNA confirmation on the presence of *Y. pestis* in human specimens has not been done. This study was conducted in Mbulu district in Northern Tanzania where plague outbreaks have recently been reported. Nine human bubo specimens were investigated for *Y. pestis* plasminogen activator gene by using polymerase chain reaction (PCR), and two were found to be positive. The two positive amplicons, together with three previously obtained PCR positive rodent samples, were sequenced using a 3130 genetic analyzer and then compared with those available in GenBank by basic local alignment search tool (BLAST). All sequences obtained from both human and rodent samples showed 99% sequence similarity to *Y. pestis* plasmid pPCP1, detected from ancient DNA, confirming the presence of *Y. pestis* in humans that possibly sourced from rodents in Tanzania.

Key words: *Yersinia pestis*, human plague, molecular detection, Tanzania.

INTRODUCTION

Plague is a deadly infectious disease that hit the Byzantine Empire, reaching Constantinople in 542 and North Africa, Italy, Spain, and the French-German border by winter 543 (Little, 2007). The etiologic agent of plague, *Yersinia pestis*, has demonstrated a remarkable ability to spread over long distances and cause intense outbreaks interrupted by long periods of silence or reduced activity. Molecular genetic investigations have indicated that *Y. pestis* spread multiple times from foci in central Asia in greatly widening swaths as human-mediated transport became more efficient (Morelli et al., 2010; Cui et al., 2013). The disease attained its current global distribution during the third pandemic, which began in 1855 in the Chinese province of Yünnan, when it was introduced into many previously unaffected countries, via infected rats on steam ships (Vogler et al., 2013). *Y. pestis* strains have historically been classified according to their ability to utilize glycerol and reduce nitrate and have been grouped into three main subtypes or biovars as Antiqua, Medievalis, and Orientalis. These biovars can be distinguished depending on their abilities to ferment glycerol and reduce nitrate (Devignat, 1951 cited in Haensch et al., 2010).

The Medievalis biovar is unable to reduce nitrates due to

*Corresponding author. E-mail: michael.ziwa@sacids.org. Tel: +255 754 888 657.

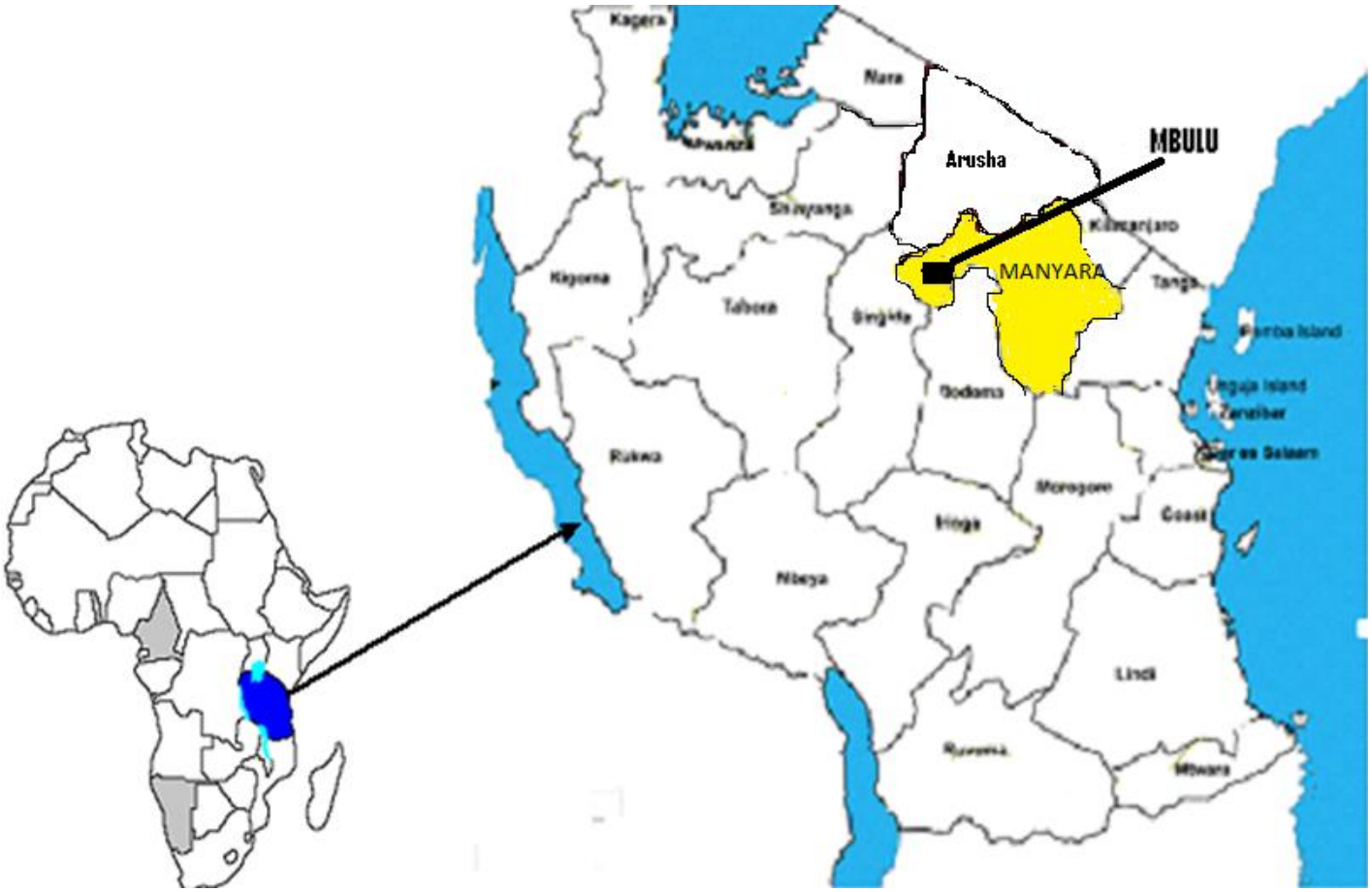

Figure 1. Map of Tanzania showing Mbulu district.

a G to T mutation that results in a stop codon in the *napA* gene (Achtman et al., 2004 cited in Haensch et al., 2010), while the Orientalis biovar cannot ferment glycerol because of a 93 bp deletion in the *glpD* gene (Motin et al., 2002; Achtman et al., 2004 cited in Haensch et al., 2010). Conversely, the Antiqua biovar is capable of performing both reactions (Haensch et al., 2010). Based on their geographic niche and on historical records that indicate the geographic origin of the pandemics, it was postulated that each biovar caused a specific pandemic (Perry and Fetherston, 1997; Wren, 2003), however, recent studies have provided direct evidence on the association of *Y. pestis* with the three historical pandemics (Bos et al., 2011; Harbeck et al., 2013). Moreover, recent collaborative efforts have provided a synthesis of old and new information describing the phenotypic and molecular diversity of *Y. pestis* (Anisimov et al., 2004; Bos et al., 2011).

In Tanzania, plague has a long history covering about 127 years, from the time it was recorded way back in 1886 believably from the Middle East (Kilonzo et al., 2005). Since then outbreaks of the disease have occurred in different parts of the country and involved large numbers of human cases and substantial case-fatality rates. During the period ranging from 1953 to 2003, 10 districts in the country reported human plague cases. Since 1980, however, only four districts (Lushoto, Singida, Karatu and Mbulu) have experienced outbreaks of the disease, and involved 8490 cases and 675 (8.0%) deaths (Kilonzo et al., 1997; 2006; Makundi et al., 2008). Despite these facts, most of the studies on plague in the country have only involved serological screening of rodents, fleas and none has utilized modern molecular tools to detect and identify *Y. pestis* directly from human patients. This study was undertaken to detect and identify *Y. pestis* from human bubo specimens in an endemic focus where cases of plague were recently reported in Tanzania (Makundi et al., 2008; allAfrica.com, 2010; Mbulu District hospital, 2011 unpublished data).

The study was conducted in Northern Tanzania in Mbulu district (Figure 1) where outbreaks of plague were reported. Mbulu is found between latitudes 3.8° and 4.5° S, and between longitudes 35° and 36° E with an altitude ranging from 1000 to 2400 m above sea level. The district

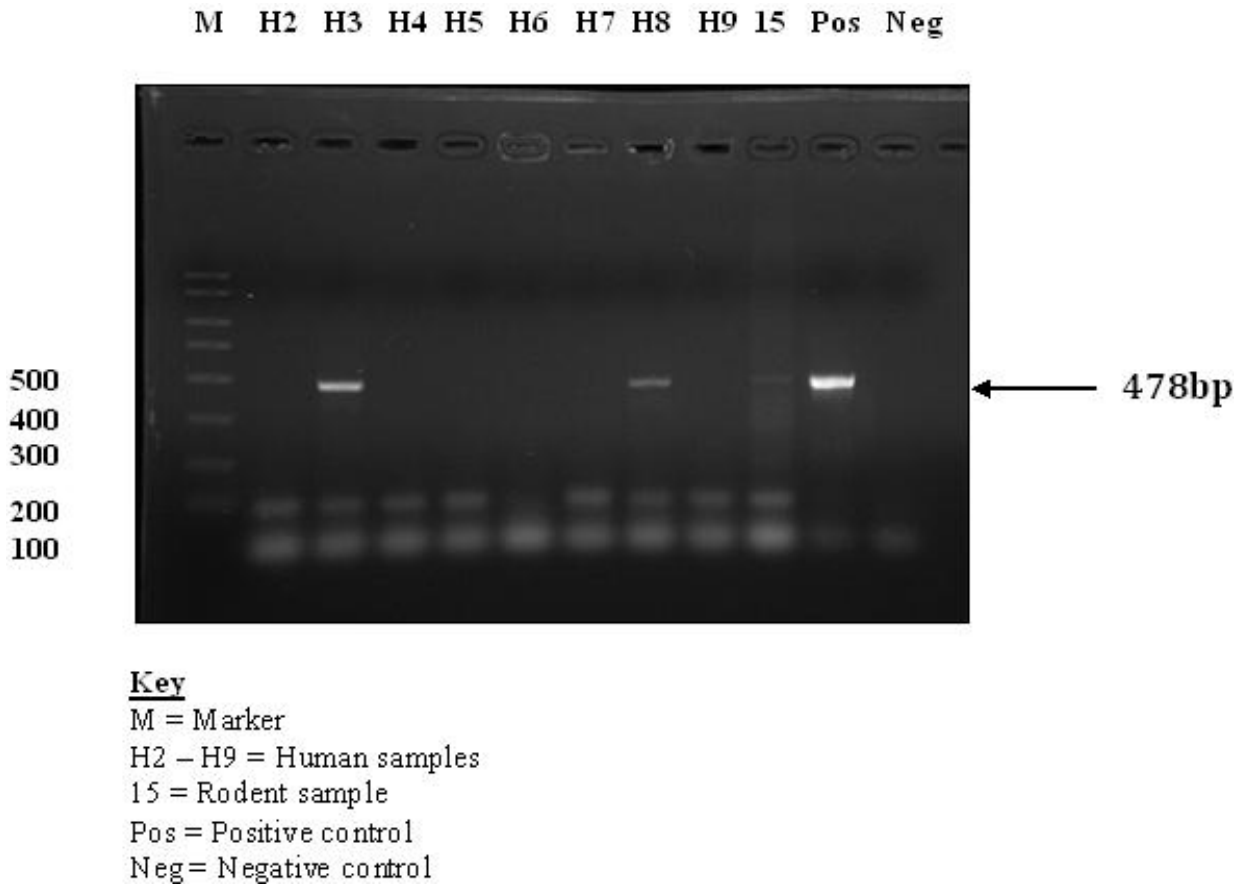

Figure 2. PCR amplification of *Y. pestis pla* gene in human bubo specimens from Mbulu district.

contains areas having semi-arid and sub-humid climate that receive annual rainfall of less than 400 mm and greater than 1200 mm, respectively (Ngowi et al., 2010).

MATERIALS AND METHODS

Bubo specimens were collected from nine patients at a local clinic during an outbreak that occurred in Mbulu district in March 2011. The bubo aspirates were inoculated in Carry Blair transport media and preserved at 4°C at Mbulu district hospital till laboratory analysis, where DNA was extracted. The volumes of the bubo aspirate were adjusted to 100 µl by adding sterile distilled water in a sterile microcentrifuge tube before DNA extraction. This was followed by the addition of the 95 µl 2X digestion buffer and 5 µl Proteinase K. The contents were mixed and incubated at 55°C for 20 min and then the total genomic DNA was extracted from the specimens using a DNA extraction kit following the manufacturer's instructions (Zymo Research, Irvine, CA, USA).

RESULTS AND DISCUSSION

To detect *Y. pestis*, specific primers previously reported (Hinnebusch and Schwan, 1993) were used to amplify a 478 bp fragment of the plasminogen activator *(pla)* gene encoded on the plasmid pPCP1. The primer sequences were *Yp* pla1: (5'- ATC TTA CTT TCC GTG AGA AG -3') and *Yp* pla2: (5'- CTT GGA TGT TGA GCT TCC TA -3') corresponding to nucleotides 971 to 990 and 1431 to 1450, respectively, of the *pla* locus sequence. The system was run in a total reaction volume of 25 µl comprising of 0.125 µl ExTaq, 2.5 µl 10XPCR buffer (Finnzyme Qy, Finland), 1.5 µl $MgCl_2$, 2.0 µl dNTP, 1 µM of forward and reverse primers each, 14.875 µl PCR water and 2.0 µl of the Template DNA. PCR amplification conditions were as follows; initial denaturation at 94°C for 1 min, subsequently 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 15 s, elongation at 72°C for 30 s and final elongation at 72°C for 5 min and then held at 4°C.

The PCR products were detected using agarose gel electrophoresis and the UV illuminator. The detected bands were subjected to sequencing. Before sequencing, the positive PCR products were purified by using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA) as described by the manufacturer. The purified DNA was then sequenced directly using a Bigdye

ready reaction kit and analyzed on a 3130 Genetic Analyzer. Some *Y. pestis* positive rodent samples from our previous work (Ziwa et al., 2013) were also sequenced and compared with those available in Genbank by BLAST search. The sequences were then aligned by using the multisequence alignment CLUSTALX version 1.8.

On PCR amplification of the *Y. pestis pla* gene the expected 478 bp segment of the *Y. pestis* plasminogen activator gene was successfully amplified from 2 human bubo specimens (Figure 2). On sequencing of the two human and three *Y. pestis pla* positive amplicons from rodents, the partial sequences were found to be 99% similar to *Y. pestis* plasmid pPCP1, detected from ancient DNA (GenBank accession number HE576987.1).

This is the first study that utilized molecular tools to detect and identify the causative agent of plague in Tanzania. The ability to link a human plague isolate to a likely source has implications for investigating natural disease events (Colman et al., 2009). In our study, we linked human disease events to the likely sources of infection, a fact that has been advanced by molecular epidemiologic techniques (Colman et al., 2009). From our results, the positive amplicons from rodents and those obtained from human samples obtained during the 2011 outbreak matched, and were all identified as *Y. pestis*. This suggests the interaction of mammalian reservoirs or flea species and humans in the infectious cycle that lead to humans contacting the disease (Ebright et al., 2003; Brinkerhoff et al., 2010).

Interestingly, the partial sequences of *Y. pestis* detected from rodents during a period of no outbreak matched with the *Y. pestis* partial sequences obtained from humans during an outbreak that occurred in 2011. This further implies that the rodent strain may be responsible for the last outbreak of plague that occurred in 2011, and that the strain is being maintained by enzootic reservoir rodents during the period of no outbreak (Riehm et al., 2011). It is unclear whether the same strain may have been responsible for the other plague outbreaks that have been reported in other parts of the country (Kilonzo and Msangi, 1991; Kilonzo et al., 2005), since molecular characterization was not performed.

Conclusion

Our preliminary results have shown that the strain of *Y. pestis* which was responsible for the human plague outbreak in northern part of Tanzania has also been confirmed in rodents. In order to provide a comprehensive picture of *Y. pestis* strains in the country we recommend isolating and characterizing the bacterium in all plague foci in the country using more discriminatory molecular techniques such as multiple-locus variable number tandem repeats (VNTR) that will clearly show the dispersion routes and position of the Tanzanian *Y. pestis* in the modern phylogenetic tree of *Y. pestis*.

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Full Length Research Paper

Comparative efficacy of *Trichoderma viride* and *Trichoderma harzianum* against *Fusarium oxysporum* f sp. *ciceris* causing wilt of chickpea

Shabir-U-Rehman[1]*, W. A. Dar[2], S. A. Ganie[2], Javid A. Bhat[2], Gh. Hassan Mir[2], Rubina Lawrence[1], Sumati Narayan[3] and Pardeep Kumar Singh[3]

[1]Department of Microbiology and Microbial Technology, AAIDU, Allahabad U.P. India.
[2]Department of Plant Pathology, AAIDU, Allahabad U.P. India.
[3]Division of Vegetable Sciences, Sher-i-Kashmir University of Agricultural Sciences and Technology, Shalimar J&K, India.

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***Fusarium* wilt (*Fusarium oxysporum* f. sp., *ciceri*) is one of the major yield limiting factors of chickpea (*Cicer arietinum*). For eco-friendly and sustainable management of the disease, two species of antagonists (*Trichoderma viride* and *Trichoderma harzianum*) and chemical fungicide (Carbendazim 50 WP) alone or in combination with farm yard manure (FYM) were evaluated against the pathogen. The study was carried out under laboratory and field conditions. *In vitro* results showed that *T. viride* and *T. harzianum* alone or in combination significantly inhibited the mycelial growth of the pathogen. Different concentrations (10, 50 and 100 ppm) of Carbendazim 50 WP showed significant inhibition in the mycelia growth, and a concentration of 100 ppm completely inhibited the mycelia growth of the pathogen. Result indicates that seed treatment with *T. viride* and *T. harzianum* reduced the wilt incidence significantly, and increased the seed germination as compared to control. Application of bio-agents alone or in combination with FYM enhanced the plant growth parameters significantly, that is, dry weight, root length and grain yield. The lone treatment with carbendazim as seed treatment significantly reduced the wilt incidence, and increased seed germination and plant growth parameters as compared to control. Results of the study show that bio-agents significantly reduced the wilt incidence, and increased seed germination and plant growth parameters as compared to chemical fungicides.**

Key words: Chickpea, *Fusarium oxysporum* f. sp., *ciceri, Trichoderma viride, Trichoderma harzianum*, carbendazim.

INTRODUCTION

Pulses are important sources of protein for vegetarian population. Chickpea (*Cicer arietinum* L.) commonly known as gram is an important pulse crop. It is the world's fourth most important pulse crop after soybeans (*Glycine max* L.), beans (*Phaseolus vulgaris* L.) and peas (*Psium sativum* L.) (FAO, 2012). In India, chickpea is ranked first in terms of production and consumption in the world. About 65% of global area with 68% of global production of chickpea is contributed by India (Amarender and Devraj, 2010). Low yield of chickpea is attributed to its susceptibility to several fungal, bacterial and viral diseases. *Fusarium* wilt caused by *Fusarium oxysporum* Schlechtend Fr. f. sp. *ciceri* (Padwick) Matuo & K. Sato, is the most important soil-borne disease of

*Corresponding author. E-mail: rshabir1@rediffmail.com.

chickpea throughout the world and particularly in the Indian Subcontinent, the Mediterranean Basin and California (Nene and Reddy, 1987). At the national level, chickpea yield losses encounter due to wilt may vary between five to ten percent (Dubey et al., 2007). Since the pathogen is both seed and soil borne, drenching with fungicides is very expensive and impractical. *F. oxysporum* f. sp. *Ciceri* is a facultative saprophytic and it can survive as mycelium and chlamydospores in seed, soil and also on infected crops residues, buried in the soil for up to five to six years (Haware et al., 1986). Therefore, integrated disease management strategies are the only solution to maintain plant health. These strategies should include minimum use of chemicals for checking the pathogen pollution, encouragement of beneficial biological agents to reduce pathogen inoculum, modifycation of cultural practices and use of resistant varieties (Bendre and Barhate, 1998).

In beneficial biological agent, *Trichoderma,* is a filamentous fungi which have attracted the attention because of their multi prong action against various plant pathogens (Harmam et al., 2004). Several modes of action have been proposed to explain the biocontrol of plant pathogens by *Trichoderma,* these include production of antibiotic and cell wall degrading enzymes, competition for key nutrients, parasitism, stimulation of plant defense mechanisms and combination of those possibilities (Cook, 1985; Harman, 2006). *Trichoderma* spp. generally grows in its natural habit on plant root surface and therefore it controls root diseases in particular (Monte, 2001; Faruk et al., 2002; Kamlesh and Gujar, 2002). The species of *Trichoderma* have been evaluated against the wilt pathogen and have exhibited greater potential in managing chickpea wilt under field condition (Podder et al., 2004). Considering these points, the present study was conducted to find out the most effective species of *Trichoderma* and fungicide against chickpea *Fusarium* wilt.

MATERIALS AND METHODS

In vitro

F. oxysporum f. sp. c*iceri* was isolated from the infected roots of chickpea plants collected at *Fusarium* infested chickpea field in the Department of Plant Pathology, Allahabad Agricultural Institute Deemed University, Allahabad, U.P. India. The fungus was cultivated on potato dextrose agar medium (PDA) and incubated for seven days at 25 ± 2°C (12/12 h light and dark cycle). The isolates were single spored and sub cultured onto PDA plates within a period of 2-3 months. Morphological characteristics of the fungal isolates were compared with standard descriptions given by Dasgupta (1988). Identification of *F. oxysporum* f. sp. c*iceri* isolates was done on the basis of cultural and morphological characteristics (Mehrota and Aggarwal, 2003). The fungal antagonist organisms *Trichoderma viride* and *Trichoderma harzianum* were obtained from the Department of Microbiology and Microbial Technology, AAIDU, Allahabad. The efficacy of antagonists against the pathogen was initially evaluated on potato dextrose agar (PDA). Discs (5 mm diameter) of seven day old culture of bio-agents were inoculated opposite to disc of the tested fungus (seven days old culture) in the same plate, both organisms were placed in such a manner that they would get equal opportunity for their growth (Dennis and Webster, 1971). The experiments were conducted with four replications\plates for each treatment, while control plates were inoculated only by tested fungus. Plates were then incubated at 27±1°C. Observation were recorded after seven days of inoculation including area covered by the *T. viride* and *T. harzianum* and the pathogen then percent of inhibition was calculated using the following formula (Vincent, 1947):

$$\text{Percent growth inhibition} = \frac{\text{Colony growth in control plate - Colony growth in intersecting plate}}{\text{Colony growth in control}} \times 100$$

Three concentrations of carbendazim (50 WP viz. 10, 50 and 100 ppm) were screened against the pathogen on PDA according to the poison food technique (Nene and Thapliyal, 1993). Four replications of each treatment along with control, maintained in completely randomized design, were incubated at 27°C. The radial growth of antagonist and pathogen was measured at 24 h intervals till 7^{th} days and the percent inhibition was calculated by applying the above formula (Vincent, 1947).

In vivo

The pathogen inoculum was prepared in potato dextrose broth (PDB) contained in 250 ml flasks and incubated in BOD incubator at 27°C for ten days. Each mycelial growth on in the liquid medium was scrapped and in 250 ml flasks containing 50 ml sterilized distilled water, these flasks were shaken on an electric shaker for 15 min at a 10 rpm. The mycelia were discarded and while spore suspension were collected separately and centrifuged at 300 rpm for one minute. The pathogen inoculums (concentration 4 x 10^6 spore\ml) were applied to nursery seedbeds at the rate of 250 ml m^{-2}, four days before seed sowing of seeds (Shabir et al., 2012; Chakraborty and Prasanta, 2001; Champawat and Sharma, 2003). The farm yard manure (FYM) was applied a week before any other treatment with a rate of 12 t ha^{-1} for each treatment. The chickpea seeds were sown in infested plots. Before sowing, the seeds as per treatment were talc based *Trichoderma* products loaded at the rate of 3.0 g kg^{-1} with both of the bio-agents (*T.* viride and *T. harzianum*) and with carbendazim at the rate of 2.5 g kg^{-1}. In the case of soil drench, *Trichoderma* was applied at the rate of 2.5 kg ha^{-1} mixed in 500 L of water. Carbendazim 50 WP was applied with a concentration of 0.25% (Shabir et al., 2013). One untreated control was also maintained. The field experiment was laid in a randomized block design with three replication for each treatment. The data with respect to percent seed germination, wilt incidence and plant growth vigor (dry weight, root length and grain yield) were recorded

RESULTS AND DISCUSSION

The result presented in Table 1 indicated that the combined effect of both antagonists (*T. viride* + *T. harzianum*) was found to be most effective (87.33%) in inhibition of Fusarium mycelia growth as compared to the control followed significantly by *T. harzianum* (73.33%) and *T. viride* (60%) (Table 1). Several studies (Jayalakshmi et al., 2009; Muhammad and Amusa, 2003; Bunker and Mathur, 2001; Shabir et al., 2013) reported that inhibition of some soil borne pathogens, including *Fusarium oxysporum* f. sp. *ciceri* by *Trichoderma* species could probably be due to the secretion of extracellular cell

Table 1. Effect of *Trichoderma* spp. and carbendazim fungicide on mycelia growth of *F. oxysporum* f. sp. *ciceri*.

Antagonist	Percent of inhibition (%)	Carbendazim (50WP)	Percent of inhibition (%)
Trichoderma viride	81.00	10 ppm	60.00
Trichoderma harzianum	83.33	50 ppm	73.33
Trichoderma viride + *T. harzianum*	87.33	100 ppm	82.22
Control	0.00 (90 mm radial growth)	Control	0.00
F test	S		S

Table 2. Effect of *Trichoderma* spp., fungicide, manure and their combinations on chickpea wilt incidence, percent of seed germination and plant growth factors in the field.

Treatment		Seed germination (%)	Wilt incidence (%)	Plant dry weight (g)	Root length (cm)	Grain yield (Kg ha^{-1})
T_0	Control	73.88	12.00	20.7	22.33	850.30
T_1	Seedbed treatment with *Trichoderma viride* + FYM	86.66	5.00	24.9	29.99	1400.00
T_2	Seedbed treatment with *Trichoderma harzianum* + FYM	91.66	4.66	25.8	30.1	1450.00
T_3	Seedbed with FYM	82.22	9.00	21.3	28.6	900.00
T_4	Seedbed drenching with *Trichoderma harzianum*	82.22	8.33	24.6	27.0	1150.00
T_5	Seed treatment with *Trichoderma harzianum*	89.99	3.66	28.5	36.1	1550.00
T_6	Seedbed treatment *Trichoderma viride*+ *T. harzianum* + FYM	87.77	5.66	23.6	31.1	1353.33
T_7	Seedbed drenching with *Trichoderma viride*	79.44	9.33	23.3	28.4	1126.7
T_8	Seed treatment with *Trichoderma viride*	89.99	3.00	30.6	36.2	1736.77
T_9	Seedbed drenching with both *Trichoderma* spp.	78.88	8.66	22.5	26.33	1550.00
T_{10}	Seed treatment with Carbendazim	78.88	8.66	22.3	24.8	996.77
	F-test	S	S	S	S	S
	CD	7.02	1.66	1.9	1.2	26.8

*Mean value of three replications.

wall degrading enzymes such as chitinase, β-1, 3-glucanase, β-1, 6-glucanase, protease, cellulease and lectin, which help mycoparasites to colonize their host. Also, inhibition of the pathogen may be attributed to the production of secondary metabolites (such as glioviridin, viridin and gliotoxin) the antagonists (Inbar et al., 1994).

Fungitoxic effect of different concentrations of carbendazim 50 WP on *Fusarium* organism was tested *in vitro* by applying poisoned food technique. *In vitro* results showed different significant levels of fungitoxicity of the different concentrations of the fungicide against the pathogen (Table 1). The highest inhibition (82.22%) of the pathogen mycelia growth was recorded from 100 ppm concentration of the fungicide, followed by 50 (73.33%) and 10 ppm (60.00%) as compared to control (90 mm radial growth) (Table 1). Sugha et al. (1995) reported that carbendazim and thiram alone or in combination were highly effective in inhibiting *in vitro* mycelia growth of the pathogen and in reducing wilt incidence under field condition. De et al. (1996) found that the coating of chickpea seed with carbendazim was more effective in reducing wilt and increasing seed yield. Gupta et al. (1997) screened six fungicides against *F. oxysporum* f. sp. *ciceri in vitro* and reported that carbendazim was the most effective inhibitor when used at a rate of 100 mg/ml.

Results show significant differences in *Fusarium* wilt disease incidence among the different treatments (T_1-T_{10}), *Trichoderma* spp., farm manure and carbendazim fungicide, when applied as seedbed treatment, seedbed drenching and seed treatment as compared to the untreated control (T_0) (Table 2). The lowest wilt disease incidence (3%) was recorded when chickpea seeds were treated with *T. viride* (T_8), followed by seed treatment with *T. harzianum* (T_5) (3.66 %). In the case of seedbed

treatment with *T. harzianum* + FYM (T_2), seedbed treatment with *T. viride* + FYM (T_1) and seedbed treatment *T. viride* + *T. harzianum* + FYM (T_6), the disease incidence was recorded as 4.66, 5.00 and 5.66%, respectively. Results demonstrated some increase in disease incidence in the other treatments, 8.33% in T4, 8.66 in T9 and T10, and 9% in T3. While the highest diseases incidence (12.0%) was recorded in the untreated control (T_0) (Table 2). Several studies reported that β-1-3 glucanase are the main skeletal polysac-charides of fungal cell wall and they also suggest chitinase and β-1-3 glucanase act as key enzymes in the lysis of phytopathogenic fungal cell wall during the antagonistic action of *Trichoderma*, hence fungal cell wall degrading enzymes of *Trichoderma* spp. are of special importance in plant defense mechanisms (Lorito, 1998; Kucuk et al., 2007; Kucuk and Kivance, 2008; Singh et al., 2008). Claude et al. (1993) showed that microorga-nisms that can grow in the rhizosphere are ideal for use as biocontrol agents, since the rhizosphere provides the front line for root against the pathogens. Merkuz et al. (2011a) reported that *Trichoderma* species are almost found in all soils worldwide.

Through the population level, the bioagents are commonly found in the rhizosphere of chickpea plants required for effective disease manage-ment. These results on the integrated management of chickpea wilt are in conformity with findings of those reported by Kolte et al. (1998), who had reported *Trichoderma* sp. as inhibitory of *F. oxysporum* f. sp., *ciceri*. Prasad et al. (2002) reported that soil application of *T. viride* and *T. harzianum*, one week before sowing, were more effective in reducing incidence of wilt and wet root rot of chickpea. *T. viride* + *T. harzianum* + *T. hamatum* were found very effective for controlling chick-pea wilt due to the synergistic effect of the three fungi.

The present study is in agreement with Padwick (1941) who reported that a species of *Trichoderma* was highly antagonistic to gram chickpea wilt pathogen under field conditions. Khodzhayan (1970) found that *Trichoderma* sp., released antibiotic substance in the nutrient media which killed *F. oxysporum* pathogen. Kirik and Steblyuk (1974) stated that *Trichoderma koningii* was strong inhibitor to *F. oxysporum* and *Fusarum culmorium*. Kaur and Mukhopadyay (1992) reported that chickpea wilt complex disease was effectively controlled by *T. harzianum* alone and in combination with fungicides. Sharma et al. (2012) and Jan et al. (2013) reported that *Trichoderma* spp., have evolved numerous mechanisms that are involved in attacking other fungi and reduce the plant diseases, enhancing plant and root growth. These mechanism include competition for space and nutrient, mycoparasitism and production of inhibitory compounds, inactivation of the pathogen enzymes (Roco and Perez, 2001) and induced resistance to crops (Kapulnik and Chet, 2000)

Results in Table 2 show significant differences in percentage of chickpea seed germination among the different applications when compared with the untreated control. It ranged from 73.88 to 91.66%. The highest percent of seed germination (91.66%) was recorded in seedbed treatment with *T. harzianum* + FYM (T_2) followed insignificantly by seed treatment with both *T. harzianum* (T_5) or *T. viride* (T_8) (89.99%). All treatment showed high seed germination as compared to control. The combination of bio-agents with FYM (T_6) showed high percent (87.77%) of seed germination followed by T_1 (86.66%), T_3 and T_4 (82.22%), respectively, while the lowest percent of seed germination (73.88%) was recorded in the control (Table 2).

The dry weights of chickpea plants were significantly differentiated among treatments of *T. viride*, *T. harzianum* and carbendazim, when used alone or in-combination with FYM (Table 2). The dry weights ranged from 20.7 to 30.6 g\plant. The highest dry weight (30.6 g) was recorded in seed treatment with *T. viride* (T_8) followed by seed treatment with *T. harzianum* (T_5) (28.5 g). In the case of combined treatments of antagonists + FYM, the dry weights were 25.8 g in T2, 24.9 g in T1, 24.6 g in T4 and 23.6 g in T6. Low dry weight of plants (22.3 g) was recorded when seeds were treated with carbendazim. While the lowest weight (20.7 g) was reported for the untreated control (Table 2).

Table 2 reveals significant differences in chickpea root length among the different treatments and applications, it ranged from 22.33 to 36.2 cm. The highest root length (36.2 cm) was observed in plots sown with *T. viride* (T_8) followed by seed treatment with *T. harzianum* (T_5) (36.1 cm), T_6 (31.1 cm), T_2 (30.1 cm) and 29.9 cm in T_1 treatment. While it was 22.33 cm in the untreated control (T_0) (Table 2).

Results demonstrated significant differences in chickpea grain yield in plots treated with the different treatments (Table 2). The grain yield ranged from 850.33 to 1736.77 kg ha^{-1}. The highest grain yield (1736.77 kg ha^{-1}) was observed in T_8, followed by T_5 (1550.00 kg ha^{-1}), T_9 (1550.00 kg ha^{-1}) and T_2 (1450.00 kg ha^{-1}). Combination of *T. viride* + FYM 1400.00 kg ha^{-1} in T1, 1353.33 kg ha^{-1} in T9 and 1150.00 kg ha^{-1} in T4) increases the grain yield significantly as compared to untreated control (850.33 kg ha^{-1}) (Table 2).

Trichoderma species has been proved to be effective against several plant pathogens (Jan et al., 2013). The present results are supported by the observations that *Trichoderma* species produces growth factors which increase the rate of seed germination (Benitez et al., 1998). Earlier studies also observed enhancing seed germination with treatment of *Trichoderma* spp., in several host pathogens systems (Kumar and Dubey, 2001). Some studies reported that the reduction in disease incidence and increase in seed germination lead to higher yield in *Trichoderma* treated seeds and soil (Dubey and Patel, 2001; Podder et al., 2004).

Srivastava (2004) reported that root colonization by

Trichoderma strains frequently enhances root growth and development. The strains of *Trichoderma* increased root development in several crops, under both green-house or field conditions (Harman et al., 2004).

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Full Length Research Paper

Evaluation of *Stropharia* sp. 1.2052 nematicidal effects against *Meloidogyne incognita* on tomato

Chuixu Kong, Chongyan Zhai, Jing Li, Jun Zhang, Keqin Zhang and Yajun Liu*

Laboratory for Conservation and Utilization of Bio-resources and Key laboratory of Microbial Diversity, Southwest China, Ministry of Education, Yunnan University, Kunming 650091, P. R. China.

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To use unique nematicidal mechanism of *Stropharia*, the strain 1.2052 was evaluated for its potential to control the root-knot nematode *Meloidogyne incognita* on tomato. *In vitro*, the inhibition rate in 24 h of *Stropharia* sp. 1.2052 isolate was 100% for second-stage juveniles of *M. incognita* and *Panagrellus redivivus*, 41.81% for *Caenorhabiditis elegans* and 99.25% for *Bursaphelenchus xylophilus*, respectively. In pot experiments, isolate 1.2052 reduced the root-knots to 68.16 to 84.19% in one month and 45.28 to 88.24% in two months after treatment, respectively. The reduction of nematodes in soil ranged from 26.39 to 61.18% compared to the negative control. There were significant efficacies for control of root knot nematodes on tomato.

Key words: *Stropharia, Meloidogyne incognita*, biological control, root-knot nematodes.

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) cause serious damages to agriculture and forest. It has been reported that plant parasitic nematodes cause annual losses of several billion of crops worldwide (Koenning et al., 1999; Perry et al., 2009). Various fungi have been tested and used to control root-knot nematodes during the last decades. The most studied fungi used and applied to control nematodes are *Hirsutella rhossiliensis* and *Pochonia chlamydosporia*. *H. rhossiliensis* is one of few endoparasitic fungi of mobile stage of nematode. Both species produce infective, adhesive conidia that may be attached to the cuticle of a passing nematode, and has shown great potential in soybean cyst nematode control (Li et al., 2008). *P. chlamydosporia* has a worldwide distribution, and has been found in nematode suppressive soils to parasitize nematode eggs (Manzanilla-Lo´pez et al., 2013). *Fusarium oxysporum* f. sp. *glycines* and *Sclerotium rolfsii* were studied for their effects on pathogenicity of *M. incognita* race 2 in soybean (Akinsanmi et al., 2003). Kiewnick et al. (2006) reported that a *Paecilomyces lilacinus* strain was selected to control *M. incognita*. *Arthrobotrys dactyloides* was tested against *M. incognita* (J2) on Tomato (Kumar et al., 2006). *Muscodor albus* was evaluated as a potential biocontrol agent against four plant-parasitic nematodes of economically important vegetable crops (Riga et al., 2008). Biological control of root-knot nematode (*M. javanica)* by *Trichoderma harzianum* BI was also investigated in greenhouse and laboratory experiments (Sahebani and Hadavi, 2008).

Species of *Stropharia* (Strophariaceae, Basidiomycota) have purple-brown to tobacco brown spores and appear in woods, grasslands, compost piles, and animal dung. *Stropharia* cultures can produce unique stellate cells, called acanthocytes. Farr (1980) has made a detailed study of the morphology and development of acanthocytes, and considered the ability to form acanthocytes as a characteristic of the whole genus (Norvell et al., 2000). Luo et al. (2006) reported that *S. rugosoannulata* showed ability to immobilize the nematodes *Panagrellus redivivus*

*Corresponding author. E-mail: lyj@ynu.edu.cn. Tel: 86-871-5032538. Fax: 86-871-5034838.

and *Bursaphelenchus xylophilus* within hours on agar plates.

The nematode-attacking activity of this fungus is carried out by spiny acanthocytes and their mechanical force is an important factor in the process. Furthermore, the growth and nematode-attacking activity of the fungus in soil were also determined, suggesting that acanthocytes are functional in soil. In the present study, mechanism of *Stropharia* sp. 1.2052 activities against a population of the root-knot nematode *M. incognita* was evaluated on tomato, in greenhouse experiments.

MATERIALS AND METHODS

Fungal culture

Stropharia sp. 1.2052 was collected from forest in Yunnan, P. R. China. The pure culture was isolated from fruiting body, identified by Luo et al. (2006), and deposited in the culture collection of Key Laboratory for Conservation and Utilization of Bio-resource, Yunnan University, P. R. China.

Solid culture of this strain was prepared on wheat grains. Wheat was soaked 24 h with tap water and 1 h with boiling water, later mixed with $CaCO_3$, glucose and sawdust (wheat, 60%; sawdust, 36%; $CaCO_3$, 2%; glucose, 2% v%). Such culture medium was transferred in 250 ml flask and sterilized at 121°C for 2 h. Inoculated cultures were incubated at 25°C for 30 days when more than 80% wheat was consumed.

Nematodes culture

M. incognita

the nematodes population was collected from tobacco in Yunnan, P. R. China. Tobacco roots with egg masses were dipped in water to remove adhering soil, cut into 0.5 to 1 cm pieces and agitated in 0.5% sodium hypochlorite solution for 2 to 3 min. Egg masses were rinsed with sterile water then placed in 0.5% sodium hypochlorite for 2 to 3 min, agitated and rinsed with sterile water (Kerry and Bourne, 2002). The eggs were incubated at 25 ± 1°C for three to five days to obtain second stage juveniles (J2) with modified Bearmann funnel method (Gray, 1984). The J2 were then used for *in vitro* and pot experiments.

Panagrellus redivivus

Nematodes were cultured on oatmeal medium (oatmeal: 20 g, water 80 ml) at 25°C for seven days, and then refrigerated at 4°C prior to use.

Bursaphelenchus xylophilus

Botrytis cinerea was cultured on a potato dextrose agar (PDA) plate at 25°C until the fungus was fully grown. The plate was then inoculated with the pine nematode, and then cultured until the fungal mycelia had been completely consumed.

Caenorhabiditis elegans

The nematodes were mutiplied in oatmeal medium at room temperature (21 to 26°C) for 6 to 7days.

In vitro experiment

Washed nematodes (80 to 100) were transferred to the PDA culture plates containing abundant well-developed hyphae of *Stropharia* sp. 1.2052. The plates were incubated at 25°C, counting the numbers of active and inactive nematodes under a stereomicroscope (50×) at different times (24, 48, 72, 96, 120 and 144 h). The nematodes were considered dead if they showed no response to physical stimuli. Uninoculated plates were used as negative control, and avermectin (0.8 µg/ml) was used as positive control. Each treatment was replicated three times, and the mortalities (%) were calculated.

Pot assay

One-month-old tomato seedlings were transplanted into 15 cm-diameter pots containing 1000 g sterilized soil (loam soil : humus soil : sand, 1:1:1,v/v). A week after transplanting, the soil around the seedlings was inoculated with *M. incognita* at 2000 juveniles per plant. Pots were arranged in a randomized block in a greenhouse at 25 ± 2°C.

Four treatments were applied: three concentrations of strain 1.2052 (50, 75 and 100 g) were incorporated with soil around the seedlings. Avermectin (0.5%, 0.02 g/pot) was used as positive control, and untreated pots were used as negative control. The experiment was completely randomized with three replicates. All pots were kept under greenhouse conditions (25 to 32°C) and watered when needed.

After 30 and 60 days, the plants were harvested. Nematode damage was measured by rating root galls on the 0 to 5 scale (0 = complete and healthy root system, no infection, 1 = very few small galls only be detected upon close examination, 2 = 25% of root system severely galled, 3 = 25 to 50% of root system severely galled and not, 4 = 50 to 75% of root system severely galled, 5 = >75% of root system severely galled, nearly no healthy roots). In addition, population of nematodes in soil, plant heights, and shoot fresh weights were determined.

Statistical analysis

Results were tested with SPSS version 17.0 by one-way analysis of variance (ANOVA). Significantly differences between treatment means were separated by using *t* test at the $P<0.05$ (Sahebani and Hadavi, 2008).

RESULTS

Effect of fungi on nematode *in vitro*

Application of *Stropharia* sp.1.2052 significantly inhibited nematodes motility *in vitro*, when compared to control in water. At 24 h complete inhibition was observed for all the J2 of *M. incognita* and for *P. redivivus*, whereas lower values were found for *B. xylophilus* and *C. elegans*, which required a longer exposure to reach the same level of inhibition (Table 1).

Effect of fungi on *M. incognita* in pot assay

Significant control of root-knot nematodes was observed

Table 1. The inhibition of *Stropharia* sp. 1.2052 on nematodes tested *in vitro*.

Test time (h)	*M. incognta* (%)	*P. redivivus* (%)	*C. elegans* (%)	*B. xylophilus* (%)	Avermectin (%)	Negative control (%)
4	46.97 ± 2.96*	25.15 ± 7.46*	8.33 ± 1.43	26.01 ± 12.13	82.28 ± 9.01**	0
12	77.47 ± 22.06*	58.68 ± 6.17**	17.64 ± 1.82	54.32 ± 2.75*	91.87 ± 14.08**	2.71 ± 0.85
24	100 ± 3.45	100 ± 5.67	41.81 ± 1.88**	99.25 ± 1.29**	93.03 ± 12.06**	6.04 ± 1.24
36	100 ± 2.78	100 ± 6.77	96.81 ± 3.13**	100 ± 2.33	93.54 ± 11.19**	9.37 ± 1.89

Means of three replicates ± SD. Values in each column followed by * one and ** two asterisks were significantly different from water control at P≤0.05 and P≤0.01, respectively.

Table 2. The control efficacy of strain 1.2052 against the *M. incognta* in pot assay.

Treatment	Dosage (g)	30 days				60 days			
		Gall index	Biocontrol efficacy (%)	J2/g of plant root	J2 in soil (% in control)	Gall index	Biocontrol efficacy (%)	J2/g of root	J2 in soil (% in control)
Negative control		2	0	29.33 ± 3.45	0	2	0	86.96 ± 6.76	0
Avermectin	0.02	0	90.2	2.33 ± 0.15	98.63	0	96.18	1.33 ± 0.45	93.01
Treat1	50	1	68.2	7.67 ± 3.78*	26.39	1	45.28	24.58 ± 2.13	61.18
Treat2	75	0	84.2	4.00 ± 2.60*	41.72	0	88.14	17.03 ± 1.33	56.77
Treat3	100	0	70.6	5.35 ± 2.34	27.88	0	67.32	20.48 ± 4.53	45.77

Means of three replicates ± SD. Values in each column followed by * one and ** two asterisks were significantly different from water control at P≤0.05 and P≤0.01, respectvely.

in pots. A dose response relationship was observed for treatments with *Stropharia* isolate 1.2052 which caused the greatest decrease of galls in one and two months, compared to positive controls. Application of *Stropharia* at lower doses resulted in lower damage reduction in one and two months, respectively (Table 2).

There were significant reductions of the *M. incognita* J2 (Table 2), however a dose-response relationship was less evident as the treatment with 75 g/pot caused the highest reduction of J2 from roots, either in one and two months, compared to negative control. Application of *Stropharia* sp. 1.2052 at 50 and 100 g had 8 and 5/g roots in one month, 25 and 21/g roots in two months, respectively. The percent reduction of nematodes in soil compared to the negative control appeared dose-dependent, and was higher than avermectin.

Differences among treatment were observed for plant height and biomass. The plants showed an increase in height after two months, with tallest plants recorded for treatment with *Stropharia* sp. 1.2052 at 75 g/pot and the shortest from the positive control. The fungus application significantly improved shoot weight over the inoculated treatment, the highest weight recorded for treatment at 75 g/pot. A dose-response relationship was also observed for *Stropharia* sp. 1.2052 applied at 50, 75 and 100 g/pot that increased the fresh root weights, compared to positive and negative controls, respectively (Table 3).

DISCUSSION

Many mushrooms, previously considered only as saprobes, are actually considered capable of taking advantage of other organisms present in their microcosm, exploited for nutritional purposes (including nematodes, plants, other fungi, and bacteria). There is a special group of nematophagous basidiomycetous fungi provided with hyphal appendages that are indispensable for their nematode-attacking ability. The appendages within the various species include hourglass-shaped adhesive knobs on members of the genus *Nematoctonus* (Drechsler, 1949, 1954), and its teleomorph *Hohenbuehelia* (Barron and Dierkes, 1977). Other appendages include the secretory cells of *Pleurotus* spp. (Barron and Thorn, 1987), the secretory appendages of *Conocybelactea* (Hutchison et al., 1996), the stephanocysts on *Hyphoderma* spp. (Burdsall, 1969; Tzean and Liou, 1993) and the spiny balls produced by *Coprinus comatus* (Luo et al., 2004). Thapa et al. (1987) reported that *Stropharia rugoso-annulata* was resistant to the mycophagous nematode species *Aphelenchoides sacchari* and *Ditylenchus myceliophagus*. However,

Table 3. Plant growth promotion of strain 1.2052 on tomato.

Treatment	Dosage (g)	Height increasing rate (%)	Fresh shoot weight (g)	Fresh root weight (g)
Negative control		78.9	12.78 ± 0.13	5.38 ± 0.34
Avermectin	0.02	74.0	8.66 ± 0.87	4.00 ± 0.29
Treat1	50	84.4	26.21 ± 1.07	10.13 ± 1.89
Treat2	75	78.8	34.25 ± 1.67*	12.68 ± 2.89
Treat3	100	79.6	32.67 ± 0.96*	19.56 ± 1.13*

Means of three replicates ± SD. Values in each column followed by * one and ** two asterisks were significantly different from water control at P≤0.05 and P≤0.01, respectvely.

Grewal (1990) found that the mycophagous nematode *Aphelenchoides composticola* was able to feed on *S. rugoso-annulata*. *Stropharia* species also have been reported to be able to immobilize the nematode *P. redivivus* and *B. xylophilus* with spiny acanthocytes and the results shown that the acanthocytes are functional in soil (Luo et al., 2006).

In the present study, a series of experiments were undertaken to further investigate the effect of *Stropharia* sp. 1.2052 against nematodes. In the *in-vitro* assay, this isolate inhibited more than 80% of motile stages of *M. incognita*, *P. redivivus* and *B. xylophilus*, except *C. elegans*. Application of *Stropharia* sp. 1.2052 to infested tomato plants in the greenhouse reduced the number of galls and final nematode population of *M. incognita* with highest efficacy either at one and two months. Such results are similar to the nematode control effects reported for many other fungi. In controlled experiments on tomato, a pre-planting soil treatment with *P. lilacinus* strain 251 was reported to reduce root galling by 66% (Kiewnick et al., 2006). When *F. oxysporum* was inoculated on soybean, the mean number of nematodes that penetrated roots decreased by 75%, when the soil was treated with *S. rolfsii* the number decreased by 78% (Akinsanmi et al., 2003). Greenhouse study shows that *M. albus* caused significant reduction (91 and 100%) of *Paratrichodorus allius* in soil, and 100% for *Pratylenchus penetrans*in bean roots and soil; 85 and 95% for *Meloidogyne chitwoodi* in potato roots; 100% for *Meloidogyne hapla* both in pepper roots and soil (Riga et al., 2008). The trapping fungus *A. dactyloides* was applied with cow dung manure, which reduced the number of root knots by 61.7 to 66.6% and of juveniles by 68.1 to 88.0% (Kumar et al., 2006).

Conclusion

Stropharia sp. 1.2052 provided effective control of nematode comparable to those reported for other fungal species. As *Stropharia* spp. are common saprophytic basidiomycete in soil, they have less chances to be pathogenic to plants and human beings, compared to other fungi. The main problems in application of *Stropharia* are related to its cultivation and form of delivering into soil. Its use as symbiont of seeds or plant roots could also be considered for future application, as well as its development in the integrated management of root-knot nematode.

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Full Length Research Paper

Effect of climatic factors on fruit rots (*Alternaria alternata* and *Aspergillus niger*) of ber (*Zizyphus mauritiana* Lamk.) and their management systems

Suresh Kumar[1*], S. L. Godara[1], S. Gangopadhyay[1], Raj Kumar[2] and Shrawan Singh[3]

[1]Department of Plant Pathology, College of Agriculture, SKRAU, Bikaner, Rajasthan, India.
[2]Krishi Vigyan Kendra, Panchmahal (Central Institute of Arid Horticulture) Godhra, Gujarat, India.
[3]Central Agricultural Research Institute, Port Blair-744101, Andaman and Nicobar Islands, India.

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The present study aimed to study influence of temperature and relative humidity on fruit rots (*Alternaria alternata* and *Aspergillus niger*) of ber (*Zizyphus mauritiana* Lamk.) and find appropriate management option during storage period. Maximum damage by *A. alternata* was observed at 25°C while *A. niger* showed maximum severity at 30°C. Positive relationship was observed between relative humidity (RH) and fruit rot by both pathogens which showed highest severity at 100% RH. The pre-and post-inoculation treatment of ber fruits with four vegetable oils, four plant extracts and four fungicides showed significant differences in effectiveness in controlling both pathogens. Neem oil and its leaf extracts significantly ($p<0.05$) reduced severity of fruit rots by both pathogens. Among chemicals, the carbendazim at 1000 ppm proved effective against *Alternaria* and *Aspergillus* fruit rot. Chemical fungicides reduced fungus growth significantly ($p<0.05$) better than botanicals; however, botanical also showed better response than control. The effectiveness of botanicals in post-harvest storage of ber can be used at commercial scale.

Key words: *Zizyphus mauritiana*, *Alternaria alternata*, *Aspergillus niger*, fruit rot, vegetable oil, botanicals.

INTRODUCTION

Ber is a tropical and subtropical fruit native to the northern hemisphere and belongs to the genus *Ziziphus* of the family Rhamnaceae and order Rhamnales. The family has 50 genera and more than 600 species (Pareek, 2001) of which the species *Z. jujube* Mill (Chinese jujube or Chinese date), *Z. mauritiana* Lamk (Indian jujube or ber) and *Z. spina-christi* (L.) wild (Christ's thorn) are the most important in terms of distribution and economic significance. Ber (*Ziziphus mauritiana* Lamk) is one of the most important fruit crops grown in arid and semi-arid regions of India (Pareek, 2001). Ber fruits are rich in dietary minerals and natural antioxidants for people in arid regions. Its fruit products are getting commercial angle in different countries, particularly for their health benefiting properties (Vithlani and Patel, 2010). Ber fruit rots have been identified as major problems next to powdery mildew in arid and semi- arid regions. Many pathogens were identified as causing agents of fruit rots of ber in storages but *Alternaria alternata* and *Aspergillus niger* were two major inciting pathogens (Sharma and Majumdar, 1993). Though, reported losses from fruit rots were 2 to 15% but this goes high during congenial climatic conditions.

Alternaria and *Aspergillus* fruit rot are the most commonly occurring fruit rots in ber. Temperature and relative humidity affect fruit rot development and establishment of pathogen infection. For this disease, the congenial temperature ranges from 20 to 30°C and high

*Corresponding author. E-mail: lorask77@gmail.com.

relative humidity (>90%). However, the temperature and relative humidity affect the severity and pathogenicity of fruit rots caused by several pathogens that is, *A*. *alternata*, *A*. *niger*, *Colletotrichum capsici*, *Drechslera australiensis*, *Fusarium moniliforme* and *F. solani* (Datar, 1995).

Application of fungicides is a common management strategy to control the post-harvest pathogens on harvested fruits. Several fungicides have been effective in checking the storage rots of fruits and vegetables caused by different fungal pathogens (Singh and Thakur, 2005; Khokhar and Tetarwal, 2012), but, use of plant derived substances is attracting consumers and researchers for health factors. Plants rich in defence factors like secondary metabolites reported to have strong anti-fungal, antibacterial properties (Reichling, 2010), therefore, could be used in post-harvest management of fruits and vegetables (Sable and Gangawane, 2010), but, information on their use was very meager, particularly on minor fruits like ber. Therefore, the present study was designed to determine congenial climatic factors (temperature and relative humidity) for two major pathogens that is, *A*. *alternata* and *A*. *niger* and to identify suitable botanicals (vegetable oils, leaf extracts) for management of fruit rots of ber in storage conditions.

MATERIALS AND METHODS

Inoculum preparation and inoculation

The pathogens were isolated from the infected ber fruits collected from various market and orchards of Bikaner district of Rajasthan. Pieces of transitional zone tissues of diseased fruits were scooped out and surface sterilized with 0.01% $HgCl_2$ solution for 1 to 2 min, followed by three washing in sterilized distilled water and transferred in petri-dishes containing potato dextrose agar medium (PDA). The crude cultures were incubated for growth at 26 ± 1°C and 95% relative humidity. The sporulating cultures were identified by microscopic examination for morphological characters of somatic and reproductive structures using reference isolates of *A*. *alternata* (Fr.) Keissler and *A*. *niger* van Tieghem. The isolated fungi were purified using single spore technique and then kept in a refrigerator on potato dextrose agar (PDA) slants for sporulation and further use (Chai et al., 1999). Healthy ripe fruits of ber were collected from main market of Bikaner, Rajasthan and washed with sterilized water. Excess water was removed and fruits were prepared for inoculation. Cork- borer wounding method was used for inoculation of the ber fruits. In brief, a hole of 2 × 2 mm size was made with sterilized cork borer and 2 mm disc cut of inoculum matrix of respective pathogens from inoculums petri-dishes was inserted in the hole by replacing the host pulp.

A piece of sterilized wet absorbent cotton was placed inside the surface sterilized polythene bags to create a micro-humidity chamber and mouth of the bags was loosely tied and incubated at 26 ± 1°C.

Environmental factors

The inoculated fruits were kept in polythene bags at four treatment levels of temperature that is, 20, 25, 30 and 35°C along with 95% relative humidity in four replications. Sterilized cotton swab placed inside the polythene bags to create a micro-humidity chamber. In another set, inoculated fruits were kept at six levels of relative humidity namely, 50, 60, 70, 80, 90 and 100% at 26 ± 1°C. The inoculated fruits kept in a desiccator which contained H_2SO_4 solution to produce a particular level of relative humidity, as suggested by Buxton and Mellanby (1934). The desiccators were sealed with grease and kept at 26 ± 1°C. The severity was recorded on the basis of percent infected area of fruit surface on 4^{th} and 8^{th} day of inoculation as described in Table 1.

Management options

Vegetable oil

Four vegetable oils namely, mustard oil, groundnut oil, castor oil and neem oil along with one control were used in four replications. In pre-inoculation, the fruits were first smeared with test oil and then inoculate after 12 h and vice-versa in case of post-inoculation treatment. The inoculated fruits were kept in the surface sterilized polythene bags with a piece of sterilized wet absorbent cotton to create a micro-humidity chamber and mouth of the bags was loosely tied and incubated at 26 ± 1°C. Fruit rot severity was recorded on 4 and 8 days after inoculation (DAI) as described in Table 1.

Leaf extracts

Another set of treatments with leaf extracts of *Azadirachta indica* A. Juss. (Neem), *Vinca rosea* L. (Sadabahar), *Withania somnifera* Dunal. (Ashwagandha) and *Ocimum sanctum* L. (Tulasi) and a control were tested against fruit rots. Leaf extracts were prepared by washing, air drying and homogenized for 5 min in sterile water (1:1, w/v). The mixture was filtered through muslin cloth and Whatmen No. 1 filter paper. In pre-inoculation treatments, the fruits were dipped in test extracts for 10 min and then inoculated. While post- inoculation treatments were applied after inoculation with *A. alternata* and *A. niger*; the interval between inoculation and treat-ment with plant extracts or vice-versa was 12 h. Proper controls were maintained and inoculation and observations were performed as per standard procedure (Mayee and Datar, 1986).

Chemical fungicides

The effectiveness of chemical control of fruit rots was tested using four commercially used fungicides namely carbendazim (50 WP), captan (50 WP), chlorothalonil (75 WP) and penconazole (10 EC) at 1000 ppm a.i. as in pre- and post- inoculation treatments against both pathogens separately. In pre- inoculation treatment, fruits were first dipped in the test fungicide for 5 min, air-dried and inoculated 12 h later while, 12 h after inoculation the treatments were applied in post- inoculation study. The experiment was arranged in completely randomized design. The procedures for inoculation and incubation of fruits and method of observations for fruit severity were recorded as described earlier (Mayee and Datar, 1986).

Statistical analysis

Data on fruit rots severity were recorded on the basis of percent fruit area infected. This was assessed with the help of an assessment key suggested by Mayee and Datar (1986). The analysis of variance was analysed using OPSTAT software (http//hau.ernet.in).

RESULTS AND DISCUSSION

Environmental factors

Normally, ber fruits are stored at room temperature (24°C) and 50 to 60% RH (Pareek, 2001) which found to be congenial for the fruit rots causing pathogens. The present study shows that the temperature significantly ($p<0.05$) affected growth of *A. alternata* and *A. niger* (Figure 1a

Table 1. Pathogen severity index.

Severity (%)	Character
0.0	No infection, ruptured fruits remain free from fungus after inoculation, fruit remain edible after removing inoculated area
0.1-5.0	Fungus growth remain only in inoculated area (<5%), fruit remain edible after removing inoculated area
5.1-10.0	Fungus showed slight growth to adjoining tissues (5 to 10%), fruits can be eaten after removing affected area
10.1-25.0	Fungus showed rapid growth and covered significant area (10 to 25%) of the infected fruits from inoculated area, fruit produce bad smell and not edible
>25.0	Most of the fruits surface (>25%) get affected and produce bad smell and not edible

and b).

Fruit rot severity of *A. alternata* was recorded to be highest at 25°C (16.0 and 27.5% at 4^{th} and 8^{th} DAI, respectively) while it was low at 20°C (9 and 16%) and 35°C (8.75 and 14.25%) (Figure 1a). In case of *A. niger*, the fruit rot severity was highest at 30°C (18.5% at 4^{th} DAI and 28.0% at 8^{th} DAI) followed by 25°C (13.25% at 4^{th} and 22.75% at 8^{th} DAI) and it was lowest at 20°C (9 at 4^{th} DAI and 15% at 8^{th} DAI) (Figure 1a and b). Similar observations were made in other crop species where 20 to 30°C reported to be optimum range for rapid development of fruit rot in chillies caused by *A. alternata*, *A. niger*, *C. capsici*, *D. australiensis*, *F. moniliforme* and *Fusarium solani* (Datar, 1995). The study suggests storing ber fruits at or below 20°C for longer time.

The severity of fruit rots by both fungi was increased with the increasing levels of relative humidity (Figure 2a and b). Maximum severity of *Alternaria* fruit rot was observed at 100% RH (16.25% at 4^{th} DAI and 24.0% at 8^{th} DAI) which was significantly higher than 3.75% at 4^{th} DAI and 7.5% at 8^{th} DAI at 50% level of relative humidity. Similar observations were also recorded in case of *Aspergillus* rot in ber where fruit rot severity was highest at 100% RH at both 4^{th} DAI (21.0%) and 8^{th} DAI (26.0%) while significantly ($p<0.05$) low at 50% RH (Figure 2a). Fruit rot severity caused by *A. alternata* and *A. niger* increased from 4^{th} to 8^{th} day of inoculation with increase in levels of humidity (Figure 2a) but it showed de-clining rate of increase (Figure 2b). It may be due to high level of initial severity by *A. alternata* and *A. niger* at higher levels of RH than 50% RH level. It might be due to enhanced conidial germination, infection and decreased host resistance at higher relative humidity (Oladiran and Iwa, 1993). However, significant reduction in relative humidity affects the quality of ber fruits in storage (Pareek, 2001); therefore, ber fruits should be stored at 50 to 60% for maintaining shelf life as well as reduce the chances of fruit rot development.

Management systems

Vegetable oils

The present study showed that pre- and post-inoculation coatings of vegetable oil on ber fruit significantly ($p<0.05$) reduced the severity of fruit rots caused by *A. alternata* and *A. niger*. Neem oil was most effective, but was at par with groundnut oil, and was significantly ($p<0.05$) better over castor and mustard oils in reducing the fruit rot severity by *Alternaria* fruit rot in pre-inoculation treatment (Table 2). The severity of *Aspergillus* fruit rot was also reduced by neem and castor oils but its efficiency was significantly ($p<0.05$) higher that groundnut and mustard oils. The post- inoculation treatments revealed that neem oil treatment was significantly better than mustard and groundnut oil in reducing the severity of fruit rot due to *A. alternata* (Table 2). Neem oil also provided effective control of *Alternaria* fruit rot as post-inoculation agent compared to other tested oils. The oils have used commercially for storage of different fruits (Sonkar et al., 2009; Khokhar and Tetarwal, 2012) and present study indentified the potential vegetable oils for extending the storage life of pathogen free ber fruits. These oils can be used as pre- or post- inoculation treatments for effective protection against both *Alternaria* and *Aspergillus* fruit rot.

Effect of leaf extracts

The biodegradable and non-hazardous nature of botanicals increased their acceptance in post-harvest management of fruit and vegetables (Srivastava and Lal, 1997; Dargan and Saxena, 2002). The results from four botanical extracts on fruit rots severity are presented in Table 3. Pre- and post-inoculation treatments of fruits with neem leaf extract was found most effective against both *Alternaria* and *Aspergillus* fruit rots. *O. sanctum* was also found to be effective treatment against

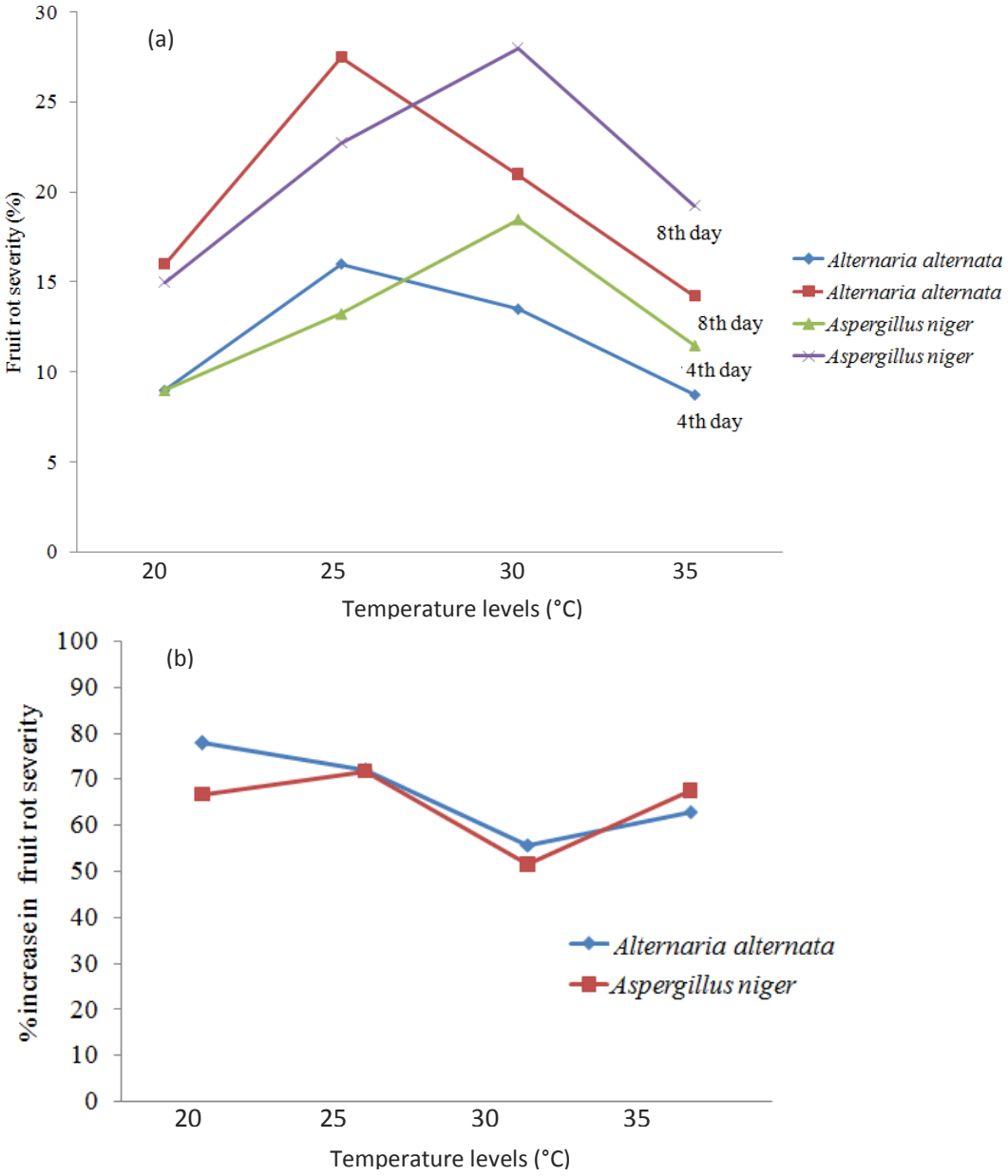

Figure 1. Effect of temperature on fruit rots by *Alternaria alternata* and *Aspergillus niger* (a) and their progress in ber (b).

these two post- harvest rots. While the performance of *W. somnifera* extract as pre- and post- inoculation treatment was relatively low against both rots. Similar reports were made by Meena et al. (2009) and Kumar et al. (2010). The variation in response of plant extracts may be due to differences in phytochemical profiles of tested plants and presence of anti-fungal compounds in leaf extracts (Cowan, 1999).

Effect of fungicides

Several kinds of synthetic fungicides had been successfully used to control the post-harvest decay of fruits and vegetables (Adaskaveg et al., 2004; Kanetis et al., 2007). But, chemical fungicides are acceptable in field level crop protection practices but their use at storage level in fruit and vegetables is discouraged for consumer and environmental benefits. However, present study tested four different fungicides against *Alternaria* and *Aspergillus* fruit rots in ber and found comparatively better than botanicals (Table 4 and Figure 3). Among organic protectants, vegetable oils found effective than plant extracts against *A. alternata* while *A. niger* but differences were non-significant. All fungicides were significantly ($p<0.05$) superior in reducing fruit rots severity as compared to control in both pre- and post-inoculation treat-

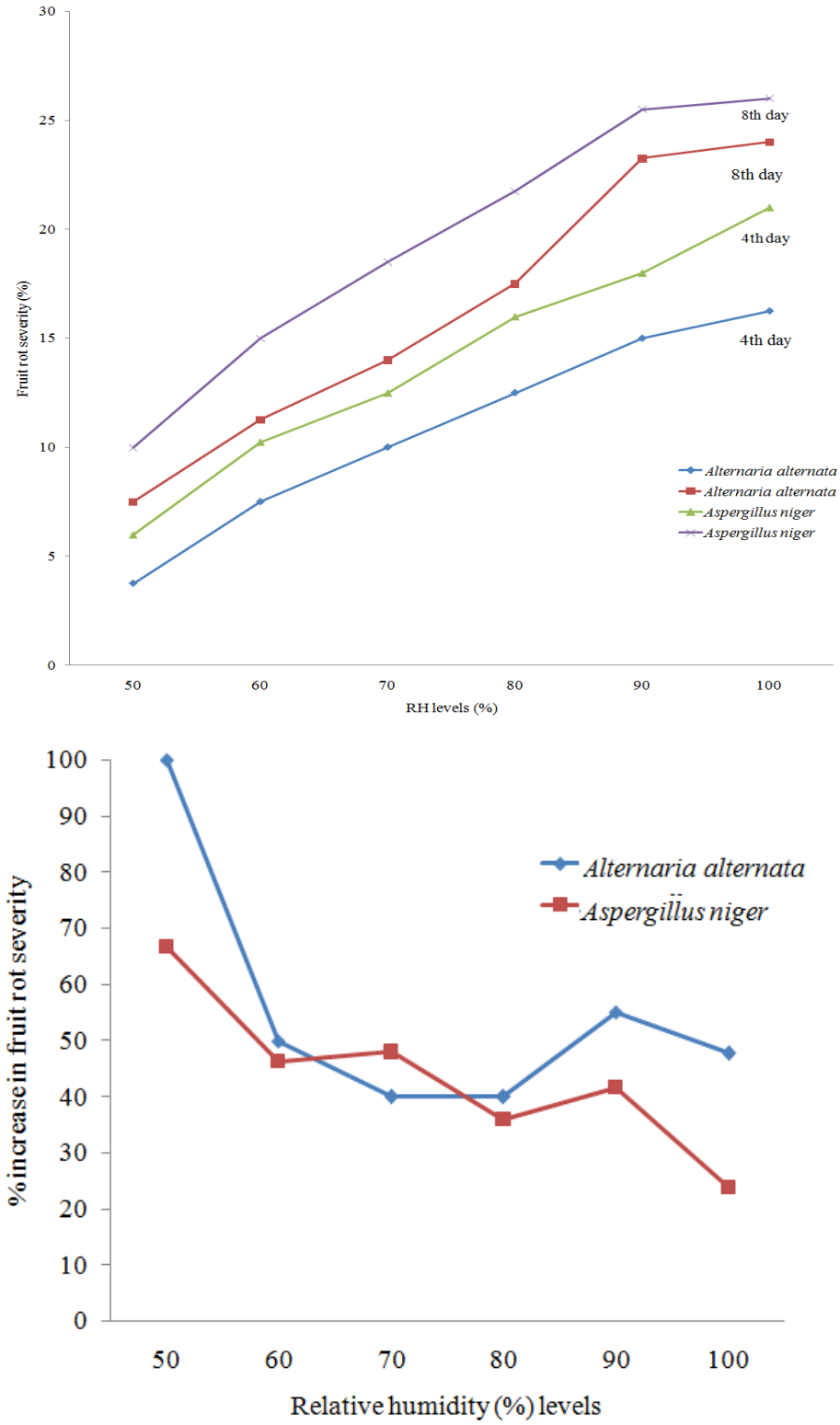

Figure 2. Effect of relative humidity (RH; %) on fruit rots by *Alternaria alternata* and *Aspergillus niger* (a) and their progress in ber (b).

ments at 4th and 8th days of inoculation. Carbendazim and chlorothalonil were found most effective against *Alternaria* fruit rot while carbendazim and penconazole for *Aspergillus* fruit rots. Similarly, different fungicides

Table 2. Effect of vegetable oils on fruit rot severity (%) by *Alternaria alternata* and *Aspergillus niger* in ber.

Vegetable oil	Pre-inoculation treatment				Post-inoculation treatment			
	A. alternata		*A. niger*		*A. alternata*		*A. niger*	
	4th DAI	8th DAI	4th DAI	8th DAI	4th DAI	8th DAI	4th DAI	8th DAI
Mustard oil	5	8	6.5	9	3.7	7.5	10	15
Groundnut oil	3	5.5	5	7.3	6.5	8	9.5	12.5
Neem oil	2.7	4	3.3	4.5	4.5	6.3	5.5	7.5
Castor oil	3.3	5.5	3.7	5.2	8	12	7.5	9.5
Control	15.5	25	17	26.5	17.5	24.5	16	25
CD (p = 0.05)*	1.9	1.9	1.5	1.9	1.1	0.8	0.9	0.8

*CD, Critical difference.

Table 3. Effect of plant extracts on fruit rot severity (%) by *Alternaria alternata* and *Aspergillus niger* in ber.

Plant extract	Pre-inoculation treatment				Post-inoculation treatment			
	A. alternata		*A. niger*		*A. alternata*		*A. niger*	
	4th DAI	8th DAI	4th DAI	8th DAI	4th DAI	8th DAI	4th DAI	8th DAI
Vinca rosea	3.5	5.7	7	11	8	11.5	10	14
Withania somnifera	6	8.5	4.3	6.3	9.5	14	8.2	12
Azadiracta indica	3	4.7	3.5	5	5	7	5.3	8.5
Ocimum sanctum	3.3	5.8	4	6	5.7	8.2	6.5	9
Control	14.5	24	18.5	27	17	26.3	16.5	28
CD (p = 0.05)*	2.1	1.4	2	1.6	1	0.9	1	0.9

*CD, Critical difference.

Table 4. Effect of chemical fungicides on fruit rot severity (%) by *Alternaria alternata* and *Aspergillus niger* in ber.

Fungicide	*A. alternata*		*A. niger*		*A. alternata*		*A. niger*	
	4th DAI	8th DAI	4th DAI	8th DAI	4th DAI	8th DAI	4th DAI	8th DAI
Carbendazim	1.3	2.5	1.0	2.3	2.3	4.5	1.5	3.0
Captan	4.0	7.5	4.8	6.0	3.0	5.3	5.5	8.5
Chlorothalonil	2.0	3.3	3.5	5.5	3.5	5.5	4.5	7.0
Penconazole	3.3	5.0	2.0	3.0	4.3	7.0	2.0	3.3
Control	16.0	26.0	16.5	25.0	16.5	27.3	17.0	27.5
CD (p = 0.05)*	1.9	1.5	1.9	1.6	1.8	1.6	2.4	2.9

*CD, Critical difference.

namely; Bavistin, Benlate, Thiobendazole, Dithane M-45, Blitox and Aureofungin have been indentified against storage rots of apple and ber (Singh and Thakur, 2005; Khokhar and Tetarwal, 2012).

A comparative analysis of three sets of anti-fungal agents indicates superiority of chemical fungicides, but their use is discouraged for consumer and environment reasons and also in organic fruits and vegetables. Therefore, the present study suggest use of vegetable oils of neem and ground nut and leaf extract of *A. indica* or *O. sanctum* as pre-infection treatment for keeping ber fruits for longer period. The same may be used for post-infection control of *A. alternata* and *A. niger* in ber and similar fruits.

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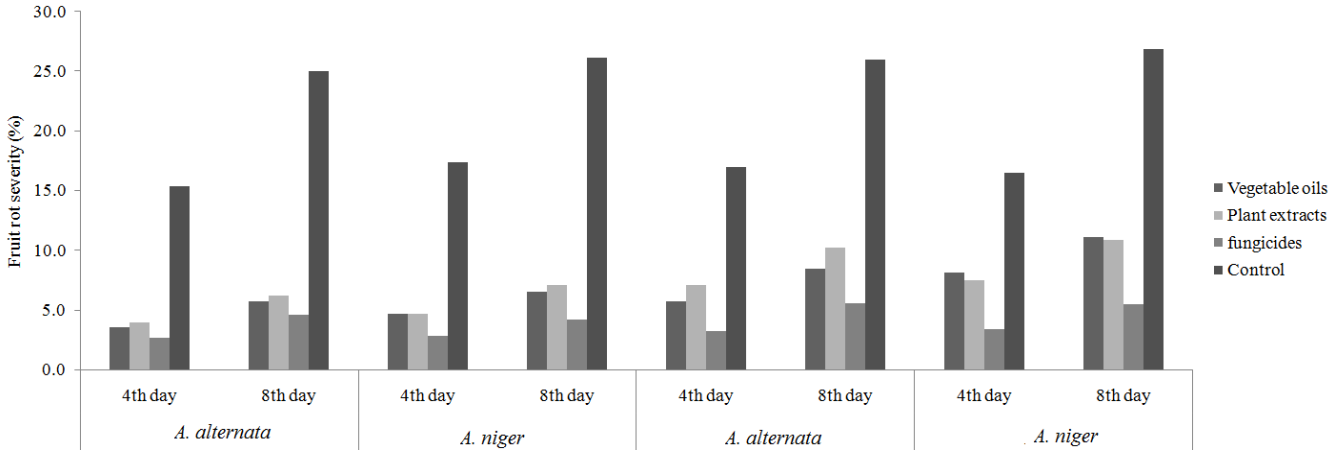

Figure 3. Relative efficacy of different treatments on fruit rot severity (mean values) of ber.

Bikaner market for sharing ber fruits for the study.

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UPCOMING CONFERENCES

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