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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

Physico-chemical surface characterization of *Bacillus cereus* spores isolated from an Algerian dairy plant

DIDOUH Nassima, CHÉRIF-ANTAR Asma, BENAMAR Ibrahim and MOUSSA-BOUDJEMÂA Boumedine*

Laboratoire de Microbiologie appliquée à l'Agroalimentaire et à l'Environnement (LAMAABE) , Ex Complexe Biomédical, Imama, University of Tlemcen, Tlemcen, 13000, Algeria.

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Bacillus cereus **is an endospore-forming bacterium frequently found in dairy products and dairy environment. In this study, the hydrophobicity and surface electrical charge of spores from fourteen (14)** ***Bacillus cereus*** **strains isolated from a dairy plant located in north-western Algeria (were studied using microbial adhesion to hydrocarbon (MATH) method, and zeta potential measurements, respectively. Spores of eleven (11) strains presented a hydrophilic character and three (3) a hydrophobic one. The spore zeta potential values for all strains were between 12.28 and −44, 51 mV. Four spore morphologies were investigated by transmission electron microscopy (TEM) after negative staining. This allowed the clear observation of an exosporium surrounding all** ***B .cereus*** **spores. The ability of spores to adhere to stainless steel was also studied and varied among strains. The presence of an exosporium was not sufficient to explain the ability of spores to adhere to stainless steel surfaces. When physico-chemical surface characters of** ***B. cereus*** **spores were compared: the hydrophobicity, the appendages length, the surface of spore and exosporium were found as the significant adhesion parameters.**

Key words: Bacterial spore, hydrophobicity, electrical charge, adhesion, transmission electron microscopy (TEM).

INTRODUCTION

Bacillus is ubiquitously present in nature, and can easily spread through food production systems. In dairy environments, *Bacillus* is part of the most commonly encountered bacteria (Salo et al., 2006; Sharma and Anand, 2002; Waak et al., 2002). Furthermore, *Bacillus cereus* is widely reported as responsible for food spoilage, and is occasionally an opportunistic human pathogen (Schoeni and Wong, 2005; Lindsay et al., 2000).

B. cereus spores are highly resistant to a large number of stresses (Lindsay et al., 2000); they have been found to account for 12.4% of constitutive biofilm microflora in a dairy plant (Matz et al., 1970; Sharma and Anand, 2002). In fact, *B. cereus* adheres easily to a range of surfaces and readily forms biofilms on food processing equipments (Faille, 2010c).

In general, *B. cereus* spores share common properties

*Corresponding author. E-mail: b.moussaboudjemaa@gmail.com. Tel: +213 551 626 216.

Table 1. Main Biochemical characteristics of *Bacillus cereus* strains used in this work.

Strains	Origin (a)	ADH (b)	GEL (c)	GLU (d)	SAC (e)	AMY (f)	Amylase	Lecithinase	Resistance to βLactams				
									CAZ	AMP	OX	KF	CRO
B. cereus 14	1	-	+	+	-	-	+	+	R	R	R	R	R
B. cereus 18	5	-	+	+	-	+	+	+	R	R	R	R	R
B. cereus 44	5	-	+	+	-	+	+	+	R	R	R	R	R
B. cereus 80	3	-	+	+	-	+	+	+	R	R	R	R	R
B. cereus 82	4	-	+	+	-	+	+	+	R	R	R	R	R
B. cereus 100	4	+	+	+	+	+	+	+	R	R	R	R	R
B. cereus 107	6	+	+	±	+	+	+	+	R	R	R	R	R
B. cereus 109	2	-	+	+	-	+	+	+	R	R	R	R	R
B. cereus 110	7	-	+	+	-	-	+	+	R	R	R	R	R
B. cereus 120	3	-	+	+	-	+	+	+	R	R	R	R	R
B. cereus 123	6	+	+	±	-	+	+	+	R	R	R	R	R
B. cereus 89	2	-	+	+	-	-	+	+	R	R	R	R	R
B. cereus103	1	-	+	+	-	+	+	+	R	R	R	R	R
B. cereus126	8	+	±	+	-	+	+	+	R	R	R	R	R

(+) positive reaction, (-) negative reaction. (a)Sources: (1): Pasteurized milk storage tank, (2): Pasteurized recombinated milk storage tank (3): Raw recombinated milk storage tank, (4): raw milk storage tank, (5): canalization of pasteurized milk; (6): canalization of pasteurized recombinated milk; (7): Canalization of raw recombinated milk; (8): Canalization of raw milk. (b) ADH: Arginine dihydrolase, (c) GEL: Gelatinase production, (d)GLU: D-Glucose utilization, (e) SAC: D-saccharose utilization,(f)AMY: Amygdalin utilization.

such as hydrophobicity and electronegativity (Andersson et al., 1998), however, some differences have been reported within the *B. cereus* group. Some spores of this group are hydrophilic (Andersson and Rönner, 1998; Tauveron et al., 2006) and the exosporium size and the length of the hair-like nap can be very different (Sylvestre et al., 2003; Tauveron et al., 2006).

In this paper, the physico-chemical characterization of fourteen (14) spores has been carried. These spores come from our *B. cereus* collection isolated from dairy equipment surfaces of a dairy plant located in Tlemcen (north-western of Algeria). The method of microbial adhesion to hydrocarbon (MATH) was used to examine the hydrophobic characteristics of *B. cereus* spores and the spore zeta potential was also measured.

On the other hand, we investigated if the exosporium and spore surfaces, the length and the number of appendages were important for spore adhesion to the stainless steel surface. This work deals with the optimization of cleaning procedures and thermochemical disinfection using detergents and disinfectants already marketed in Algeria.

MATERIALS AND METHODS

Origin of *B. cereus* strains and stock spore production

Samples came from inner tanks surfaces of pasteurized and unpasteurized local milk, tanks of pasteurized and unpasteurized recombined milk and from packaging lines.

Fourteen *B. cereus* strains from our collection of 155 strains isolated in 2010-2012 from dairy plant processing lines located in Tlemcen (north-western of Algeria) were analyzed in this study (Table 1). All the equipment was sampled after the cleaning and sanitizing procedures.

Biochemical identification of *B. cereus* was done by determination of respiratory enzymes: catalase, cytochrome-oxidase (TMPD test) and the reduction of nitrate. Additional biochemical tests for β-galactosidase (ONPG), ornithine decarboxylase (ODC), lysine decarboxylase (LDC) and the arginine-dihydrolase (ADH) activity, production of H_2S, use of the citrate, production of indole and Voges-Proskauer reaction, gelatin liquefaction and degradation of some sugars were performed. These tests were done using the API20E plate (bioMerieux SA, Lyon, France, test kit) (EL Sersy and Mohamed, 2011).

We also looked for: Extracellular hydrolytic activity as for amylolytic and proteolytic activity:, namely the search of the caseinase activity, and the determination of lipolytic activity (lecithinase test). Resistance to Four β-lactam antibiotics: ceftazidime (CAZ), ampicilline (AMP), céfalotine (KF), oxacilline (OX) and ceftriaxone(CRO) (Bio-Rad- Exosporium structure was observed by transmission electron microscopy (Table 1).

Sporulation was induced by adding $MgSO_4$ (40 ppm w/v) and $CaCl_2$ (100 ppm w/v) in nutrient agar, and followed by microscopic observations. When at least 90% of spores were observed (in general after 4 to 6 days at 37°C), the culture was harvested and subsequently washed with sterile distiller water (three times) then centrifuged (4000 rev/min) for 15 min in an Eppendorf Centrifuge 5810 R (Leguerinel et al., 2000).

The spore suspensions were stored at 4°C in sterile distiller water until use. Before each experiment two additional washes with sterile distiller water were performed.

Determination of physico-chemical properties of spores

In order to characterize the spore hydrophobic property, a MATH

partitioning method was used, based on the affinity of spores to an apolar solvent, that is, hexadecane (Sigma). The surface hydrophobicity of bacterial cells has been previously determined by several methods based on the precipitation of cells by salts (Leguerinel et al., 2000), hydrophobic interaction chromatography (Doyle et al., 1984; Smyth et al., 1978), and adherence to various liquid hydrocarbons including hexadecane (Craven and Blankenship, 1987; Kutima and Foegeding, 1987; Doyle et al., 1984; Rosenberg et al., 1980) but the hexadecane-aqueous partition system used in our work is one of the simplest and fastest methods described.

Spore suspensions in a saline solution (0.85% NaCl solution) were adjusted to an absorbance of 0.5 to 0.6 at 600 nm (A_0) in glass tubes (10 x 75 mm). Three milliliter aliquots of each spore suspension and 500 µL of hexadecane were vortexed four times ranging from 5 to 150 s and left to settle for 30 min, to allow complete separation into two phases. The absorbance at 600 nm of the aqueous phase was measured (A_t), and then $\frac{At}{A0} \times 100$ was plotted against the vortexing time (s). The initial slope, giving the initial removal rate (R_0) from the aqueous suspension, is related to the hydrophilic/hydrophobic spore character. A spore was considered to be hydrophobic when (R_0) fell between −4.0 and −6.0 and to be highly hydrophobic for lower values.

The spore zeta potential was measured using a zetameter (ZetaCompact, CAD Instrumentation, France). This was determined from the electrophoretic mobility using Helmholtz–Smoluchowski equation. For this purpose, spores were suspended in 1 mM KNO_3 to obtain around 50 spores per analysis. The pH was adjusted to values ranging from 3 to 9, with HNO_3 1 mM or KOH 1 mM. Trials at pH 2.86 were performed directly in HNO_3 1 mM. Each sample was analyzed in duplicate (10).

Transmission electron microscopy (TEM)

Spores were adsorbed onto Formvar-coated grids (EMS, 22400) and examined after negative staining with 2% w/v uranyl acetate (EMS, G100H-Cu) on a Hitachi H7500 electron microscope at an accelerated voltage of 80 kV. About 50 TEM pictures were taken for each spore.

Test of spore adhesion to stainless steel coupons

In order to determine the relationship between the physico-chemical properties (hydrophobicity and electrophoretic mobility) and adhesion, spores were analyzed for their ability to adhere to stainless steel coupons in static conditions.

The adhesion of spores from four selected *B. cereus* strains to stainless steel was tested on coupons (15 × 45 mm, AISI 304 L, bright annealed), which were filled-up by vertical immersion for 4 h in an aqueous spore suspension (10^5 spores/mL), and then quickly rinsed with sterile water. The fouled coupons were subjected to ultrasonication in 10 mL Tween 80 2% (v/v) during 5 min, (Ultrasonic bath, Deltasonic, France). The detached spores following sonication were enumerated on nutrient agar (Bio-Rad Laboratories, France), after 48 h at 30°C (Faille et al., 2013). All experiments were repeated three times

RESULTS AND DISCUSSION

In the present study, we evaluated the surface physico-chemical properties of spores from fourteen *B. cereus* strains and the adhesion abilities of 4 representative isolates (2 with hydrophobic and 2 with hydrophilic spores) on the stainless steel surface.

Table 2. Hydrophobicity and zeta potential of fourteen *Bacillus cereus* spores isolated from an Algerian dairy plant.

Strains	Hydrophobicity (s^{-1})	Zeta potential (mV)
B. cereus 014	2.34±0,58	-26.630±1,99
B. cereus 018	3.53±0,08	-26.990±2,47
B. cereus 044	2.16±0,70	-19.360±3,96
B. cereus 100	2.10±0,38	-37.055±1,067
B. cereus 107	1.79±0,95	-32.300±1,51
B. cereus 109	0.107±0,20	-20.806±0,42
B. cereus 110	5.32±1,07	-28.085±10,38
B. cereus 120	1.34±1,28	-31.225±3,32
B. cereus 123	4.34±0,83	-26.590±4,46
B. cereus 80	1.17±0,67	-19.315±0,091
B. cereus 82	2.72±0,59	-20.910±3.74
B. cereus 89	2.85±1,02	-27.065±4,49
*B. cereus*103	2.05±0,1	-12.285±1,18
*B. cereus*126	0.250±1,58	-44.510±7,28

The results on the spores hydrophobic/hydrophilic character estimated by MATH assay and their zeta potential are given in Table 2, Figures 1 and 2. From the values obtained in this work, the isolates were classified in three groups:

Group 1: Highly hydrophilic spores (14.29%) including *B. cereus* 109 spores with an initial removal rate of −0.107 s^{-1} and *B. cereus* 126 with initial removal rates around −0.25 s^{-1}.

Group 2: Moderate hydrophilic spores (64.29%) including spores from 9 B. *cereus* strains with initial removal rates between 2.05 s^{-1} (*B. cereus* 103) and 2.85 s^{-1} (*B. cereus* 89).

Group 3: moderately hydrophobic (21.43%) including spores from 3 *B. cereus* strains as indicated by the initial removal rate ranging from −3.53 s^{-1} (*B. cereus* 18) to −5.32 s^{-1} (*B. cereus* 110).

The spore electric charge characterized by the zeta potential indicated a clear electronegative character of all strains at pH 7.0. However wide variations were observed between strains (zeta potential ranging from −12.28 to −44, 51 mV). The less negative charge was -12.28 (strain 103). In conclusion, this data set showed no correlation between the hydrophilic/hydrophobic character and spore electric charge (R^2=0.0137).

In this study hydrophobicity and surface electrical properties of *B. cereus* spores were in the range or lower, than that observed in previously published data. Indeed, Ankolekar and Labbe (2010) found that the values of hydrophobicity ranged from 55.6 to 14.1% and those for Zeta potential from -8.18 to -26.8. Instead Faille et al. (2010 a), found that the values of hydrophobicity ranged from 9 (≈45%) to 0.5 (≈2.5%) and Zeta potential from

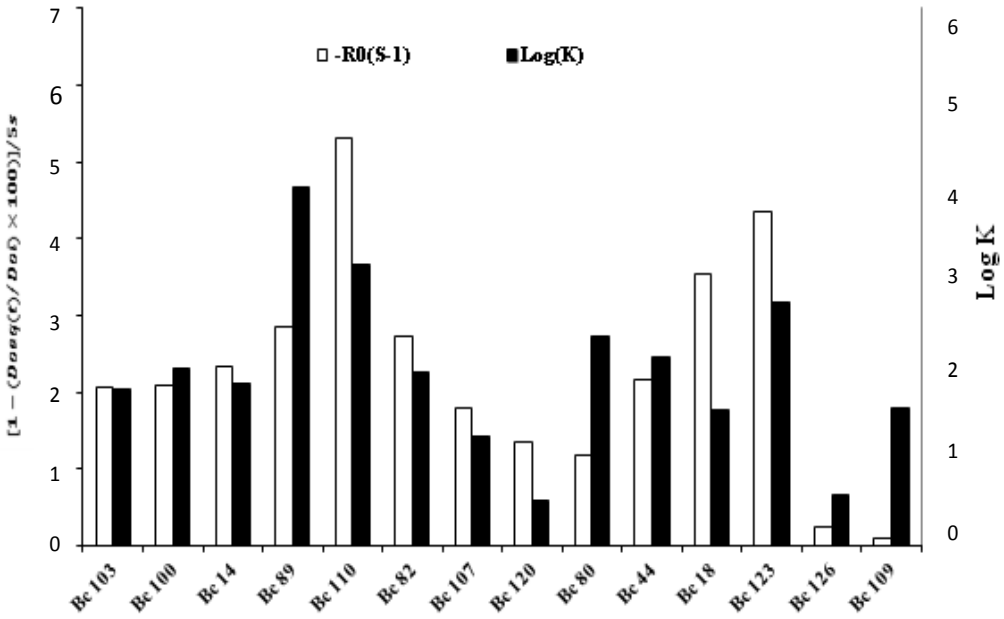

Figure 1. Affinity for hexadecane of spores of fourteen *B. cereus* strains isolated from an Algerian dairy plant. Left axis represent the initial removal rate R0 from the aqueous suspension and right axis represent the energy of affinity of hexadecane.

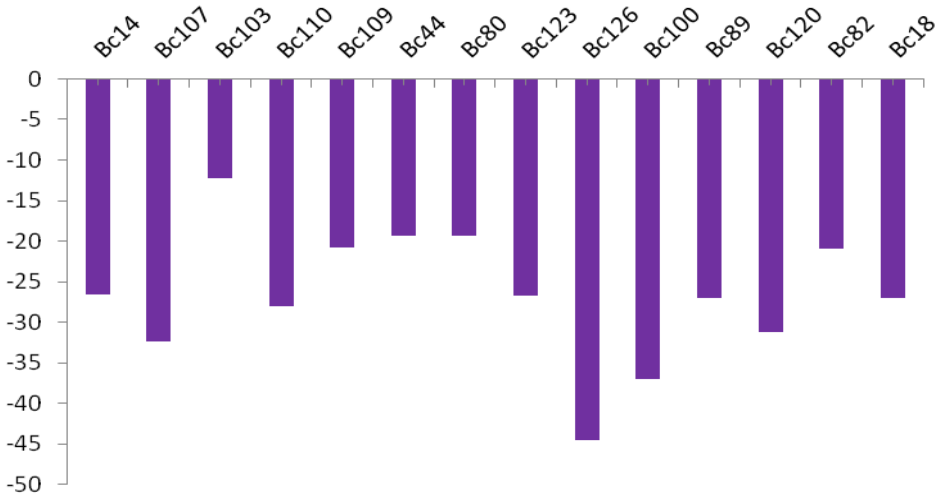

Figure 2. Zeta potential of spores of fourteen *B. cereus* strains isolated from an Algerian dairy plant.

−17.61 to −46.81, and Buhr et al. (2008) found that the values of hydrophobicity ranged from 92.2 to 12.7. (Ankolekar and Labbe, 2010; Buhr et al., 2008; Faille et al., 2010b).

The observation of whole spores from 4 strains (*B. cereus* 110 and *B. cereus* 123 had hydrophobic spores and *B. cereus* 82 and *B. cereus* 109 had hydrophilic ones) by electron microscopy and the examination of the pictures for each spore showed that *B. cereus* whole spores shared a common architecture such as the presence of an exosporium surrounding the spores. The presence of appendages on spores was also examined (Figure 3).

For the strain *B. cereus* 110, the hydrophobic character was found to be linked to the presence of an exosporium in agreement with the work of Koshikawa et al. (1989). However, our results indicated that *B. cereus* 109 and *B. cereus* 82 spores, being hydrophilic, were surrounded by

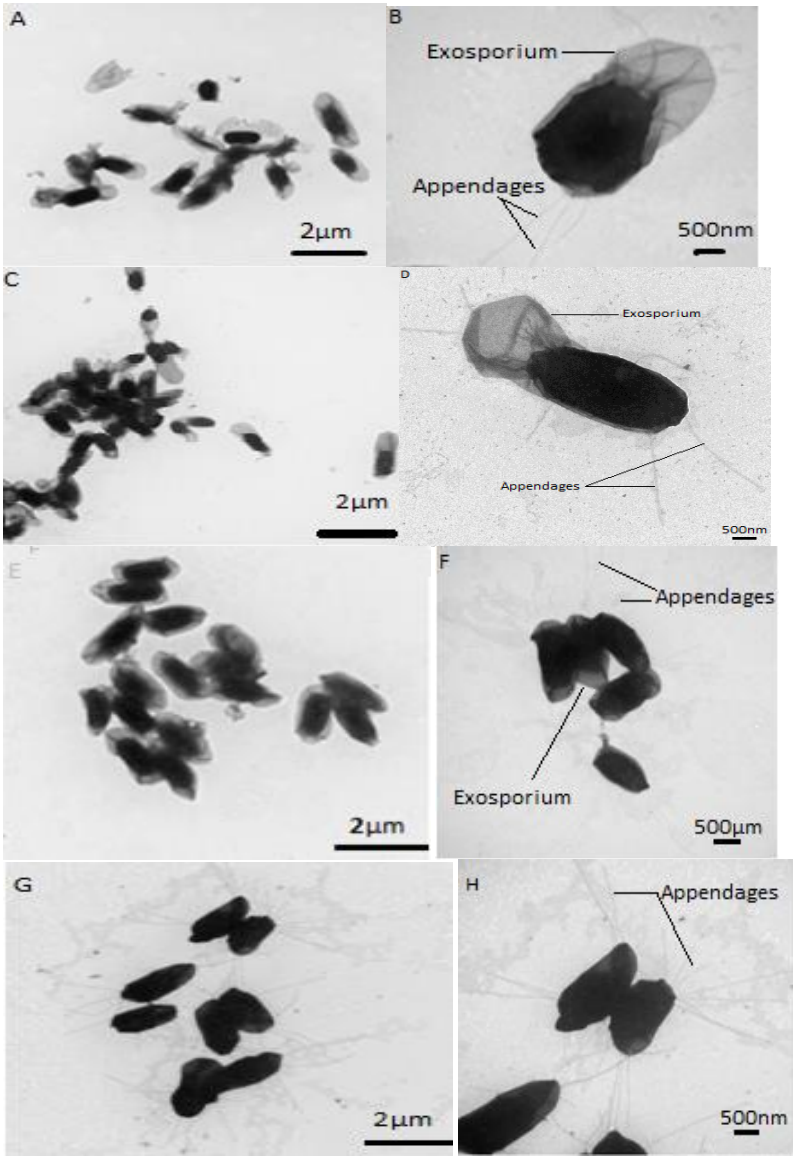

Figure 3. Transmission electron microscopy of spores after negative staining. (A, B): *B. cereus 82*; (C, D): *B. cereus109*; (E, F): *B. cereus 110*; (G, H): *B. cereus 123*.

an exosporium. It has been suggested that the increased hydrophobicity of bacterial spores is due to the relative abundance of proteins in the outer coats and exosporium when compared with peptidoglycan on Gram-positive vegetative cell surfaces (Henriques and Moran, 2007; Doyle et al., 1984; Takumi et al., 1979; Matz et al., 1970).

Kozuka and Tochikubo (1985) reported chemical properties of the main component of *B. cereus* IAM1110 appendages. They suggested the appendages may not have strong hydrophobic properties in comparison with the exosporium.

Appendages have a spiral structure (Faille et al., 2010a) and are a common but not an universal feature of a *B. cereus* group. The number and length of spore appendages of the *B. cereus* group is species-associated. Wijman et al. (2007) mention that the variation of number and length of spore appendages can be due to their fragility and loss during the preparation operations (Wijman et al., 2007). We observed such appendages on the surface of spores of every strain. Yet, the number of appendages observed in our work varied among strains ranging from 5.32±2.76 to 7.81±4.24 (means of 50 pictures of each strain) and the length varied from 0.50 to 3.74 µm as determined by Image J software (rsbweb.nih.gov/ij/download.html). Similar values have been reported by Ankolekar and Labbé

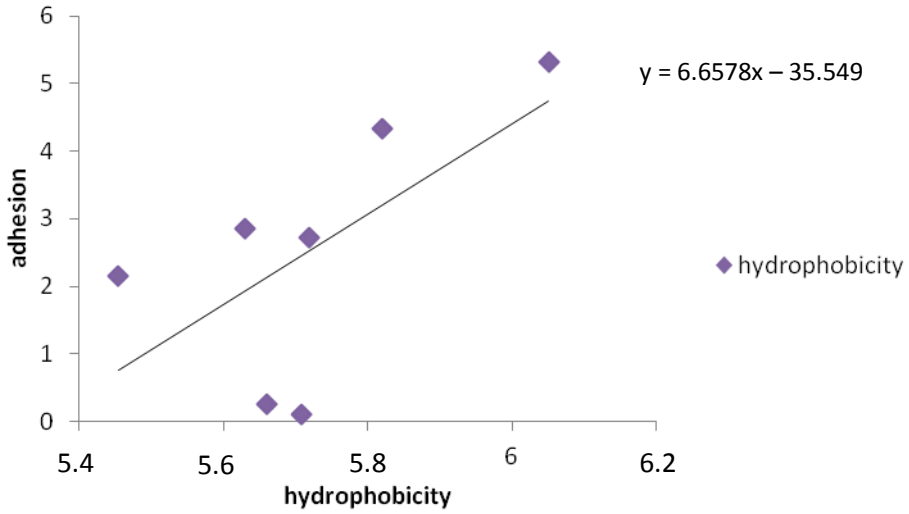

Figure 4. Relation between bacterial adhesion and hydrophobicity of spores from seven *B .cereus* strains isolated from an Algerian dairy plant (spores are: *B. cereus* 107, *B. cereus* 109, *B. cereus* 110, *B. cereus* 120, *B. cereus* 123**,** *B. cereus* 082 and *B. cereus* 018 and *B. cereus*103).

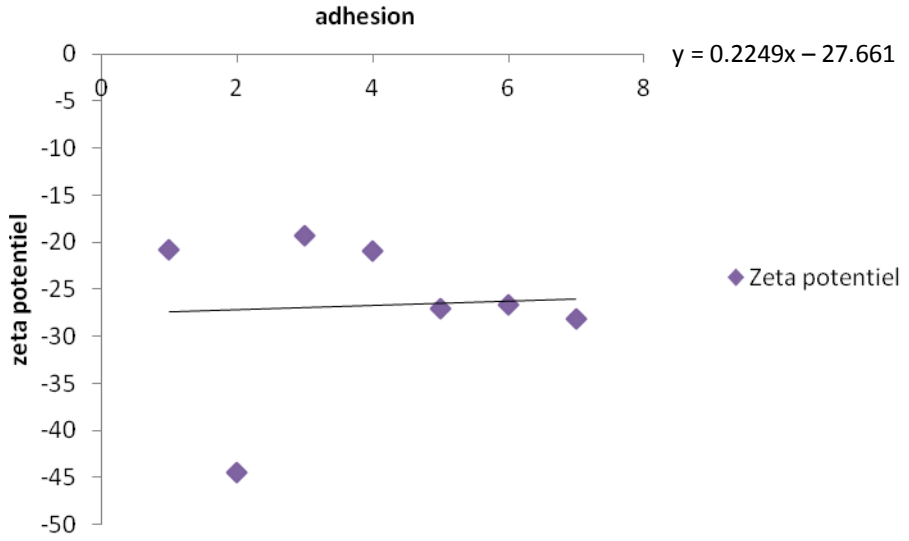

Figure 5. Relation between electrophoretic mobility and adhesion capacity of spores from seven *B. cereus* strains isolated from an Algerian dairy plant.

(2010) and Faille et al. (2010b). For each strain the appendages were peritrichous.

Our results indicated that the physical-chemical characteristics of *B. cereus* are independent on the source sampling (Table 1 and Figures 4 to 6).

The correlation between bacterial cell surface hydrophobicity and adhesion capacity of spores is given in Figure 4. A linear correlation was found between bacterial hydrophobicity and adhesion (y=6.66x + 35.55).

The relation between bacterial cell surface charge and bacterial adhesion capacity of spores is given in Figure 5. No correlation was found between bacterial zeta potential and adhesion (y = 0.22x – 27.66).

Figure 6 illustrates the relation between bacterial electrophoretic mobility and spores hydrophobicity and shows also that no correlation was found between bacterial zeta potential and hydrophobicity (y = 0.66 x – 28.17).

In accordance with previous works, spores with lower charge have a higher adhering ability to surfaces

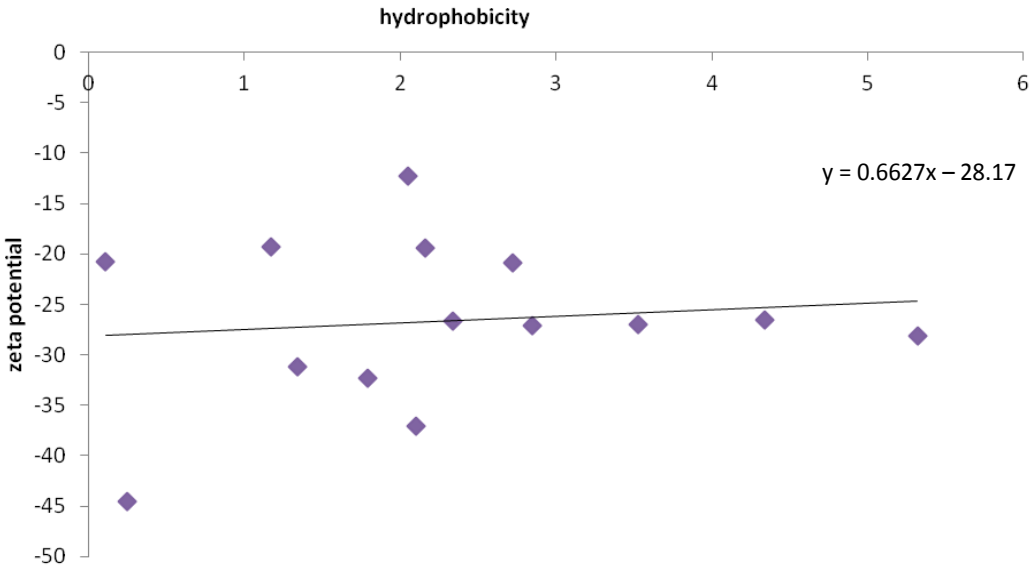

Figure 6. Relation between hydrophobicity and zeta potential of *B. cereus* spores from fourteen strains isolated from an Algerian dairy plant.

Table 3. Relation between some spore surface characteristics and number of adherent spores of *B. cereus* (mean of three trials).

Strains (a)	Surface of exosporium (µm^2)	Surface of spore (µm^2)	Number of appendages	Length of appendages (µm)	Number of adherent spores/cm^2
B. cereus 110	0.48	0.39	6±3	0.58	1.12×10^6
B. cereus123	0.43	0.36	5 ±2	0.53	6.60×10^5
B. cereus 82	0.53	0.41	7± 2	3.74	5.23×10^5
B. cereus 109	0.56	0.43	8 ±4	0.50	5.10×10^5

[a]*B. cereus 110* and *B. cereus 123* are hydrophobic and *B. cereus 82* and *B. cereus 109* are hydrophilic.

(Hüsmark and Rönner, 1992; Giarouris et al., 2009), and hydrophobicity was shown to play a major role in spore adhesion (Faille et al., 2013). On the other hand, the spores are covered with long appendages and these promote adhesion (Stalheim and Granum, 2001; Smirnova et al., 1989). Husmark and Rönner (1992) found that adhesion of *B. cereus* IAM1110 spores after sonication, which removes the appendages is around 2.5 time less than adhesion of *B. cereus* whole spores.

Due to the relatively high hydrophobicity, spore adhesion is especially high to hydrophobic materials such as stainless steel, which is commonly used in dairy processing equipment. *B. cereus* spores present a remarkable ability to adhere firmly to various inert materials (Seale et al., 2008).

The work of Klavenes et al. (2002) on the attachment of *B. cereus* spores to stainless steel surfaces shows that in contrast with the results from the static conditions, the dynamic conditions gave unexpected results. One possible reason for this might be that the appendages promote the initial adhesion of the spores, but when finally attached, the appendages serve no further purpose and other adhesion mechanisms dominate. Another explanation could be that spores with appendages aggregate more easily or get scrambled into each other, making large clusters of spores which are more easily removed from the surface in dynamic conditions. Some controversy as to their role in adhesion persists (Seale et al., 2008). Cleaning agents that degrade appendages already exist and could possibly be developed further if the appendages are found to be critical in the adhesion phenomenon (Stalheim and Granum, 2001).

Results given in Table 3 indicated that the adhesion was affected by the length of appendages while Faille et al., (2010b) found that the adhesion of spores of *B. cereus* is due to the number of appendages. Over the spore surface and exosporium and little more, the adhesion is strong.

According to the work of Rönner et al. (1990), the most hydrophobic spores (measured by the hydrophobic interaction chromatography method) are able to adhere in a much larger extent to the hydrophobic surfaces (Rönner et al., 1990). *B. cereus* 110 and *B. cereus* 123 are hydrophobic and strongly adhering to the stainless steel.

Conclusion

The spores' surface characterization showed that two-thirds of our spores were moderate hydrophilic and the spore electric charge characterized by the zeta potential indicated a clear electronegative character of all strains at pH 7.0; however, huge variations were observed between strains.

Our results show also that there is no correlation between adhesion and Zeta potential characters. A weak correlation was found between bacterial hydrophobicity and Zeta potential and a real correlation was found between bacterial hydrophobicity and adhesion.

Also, the ability of spores to adhere to stainless steel surface was essentially related to the differences in the length of the appendages, the surface of the exosporium and spore and electrical charge.

These data are very important. In fact we can use chemical agents that degrade appendages or modify the surface properties (enzymes or surfactants). We can also try physical treatments as ultrasonic cleaners to improve cleaning and disinfection strategies.

Conflict of interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

Prevalence and distribution of methicillin resistant *Staphylococcus aureus* (MRSA) among laboratory science students and laboratory staff from a single hospital in North Saudi Arabia

Mohammed Suliman Al-Mogbel

Molecular Diagnostics and Personalized Therapeutics Unit, College of Applied Medical Sciences, University of Ha'il, Kingdom of Saudi Arabia.

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Staphylococcus aureus **causes morbidity and mortality in both community and hospital settings. Methicillin resistant *S. aureus* (MRSA) is being increasingly reported globally. This study aimed at finding out the prevalence and the distribution of the community-associated MRSA (CA-MRSA) or healthcare-associated MRSA (HA-MRSA) among 150 subjects. Of the 150 subjects, 125 were students of laboratory science and 25 were laboratory staff. Nasal swabs were collected aseptically and cultured using standard microbiological protocols. Antibiotic susceptibility was done according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Methicillin resistance was detected by resistance to oxacillin and cefoxitin and confirmed by detecting *mecA* gene. Strain typing of MRSA strains was done by pulse field gel electrophoresis (PFGE). The distribution of CA-MRSA from all MRSA isolates were, 100% (2/2) from the first year laboratory science students, 100% (2/2) from the second year laboratory science students, 100% (2/2) from the third year laboratory science students, 67% (2/3) from the fourth year laboratory science students, 43% (3/7) from fifth year students and 40% (4/10) from the hospital staff, respectively. The PFGE results showed that out of total 26 MRSA isolates, there were two major groups; 15 were found to be of one group, consisting of all CA-MRSA with SCC*mec* type IV; and 11 isolates were of second group, HA-MRSA with SSC*mec* type III and IIIA on the other group. Additionally, 100% (15/15) and 20% (3/15) CA-MRSA isolates were found positive for Panton-Valentine leukocidin (PVL) and toxic shock syndrome toxin-1 (TSST-1), respectively. Furthermore, the CA-MRSA isolates showed a higher susceptibility pattern to non-β-lactam antibiotics as compared to HA-MRSA. Our study reports a high percentage of CA-MRSA isolates among the healthcare workers who have lesser or no exposure to the hospital environment as compared to those with high exposure. Also, the genetic relatedness, presence of Panton-Valentine leukocidin (PVL) and identical antibiogram of CA-MRSA makes this study interesting, as carriage of these isolates in the laboratory students of hospital setup may play a key role in the epidemiology and pathogenesis of infection in the hospitals in future.**

Key words: Methicillin resistant *Staphylococcus aureus* (MRSA), healthcare workers, antibiotic susceptibility, molecular typing.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a potential harmful pathogen associated with several infections like, bacteremia, infective endocarditis, sepsis, toxic shock syndrome, and skin and soft tissue infections

(van Hal et al., 2012). Infections caused by MRSA lead to excess morbidity and mortality among hospitalized patients, exhibiting a serious healthcare issue worldwide (Ippolito et al., 2010). The first MRSA strain was isolated in hospitals in the United Kingdom in 1961 and thereafter, reported worldwide rapidly (Abujheisha, 2013). The resistance to methicillin among MRSA was reported to be due to *mecA* gene which encodes a penicillin-binding protein that has got a reduced affinity towards methicillin. As a result of production of this unique penicillin-binding protein, methicillin cannot bind to the bacterial cell efficiently, which in turn results in reduced capacity of methicillin to inhibit bacterial cell-wall synthesis. The *mecA* gene was shown to be present on a mobile genetic element called staphylococcal chromosome cassette mec (SCC*mec*) (Borbón-Esquer et al., 2014). Currently, MRSA is a cause of concern for the healthcare society globally because this strain has acquired resistance to several classes of antimicrobial agents, therefore, it commonly exhibits the multidrug resistance (MDR) phenotype, thus poses a continuous threat for failure of antimicrobial therapy (Cadilla et al., 2011). Initially, MRSA infections were reported in the community among individuals who had had recent exposure to health-care settings or had been in close contact with MRSA-infected individuals (Lowy et al., 1998), and therefore, MRSA was considered to be primarily a health-care-associated threat until the late 1990s. During mid-90's, a sudden change in the MRSA target population occurred, and healthy individuals in the community developed MRSA infections rapidly and these infections were called CA-MRSA (DeLeo et al., 2010; Otto, 2010). The first case of CA-MRSA was reported in 1993 from Australia (David and Daum, 2010) and shortly thereafter, CA-MRSA cases were reported worldwide (Chatterjee and Otto, 2013).

The CA-MRSA strains have been distinguished from their HA-MRSA counterparts by different molecular methods. HA-MRSA strains carry a relatively large staphylococcal chromosomal cassette *mec* (SCC*mec*) belonging to types I, II, or III. All these cassettes contain the *mecA* gene signature, which is nearly universal among MRSA isolates. HA-MRSA strains are often resistant to many classes of non-β-lactam antimicrobials. In contrast, CA-MRSA isolates carry smaller SCC*mec* elements, most commonly SCC*mec* type IV or type V (Miller et al., 2008), are often susceptible to many classes of non-β-lactam antimicrobials and carry the genes for the virulence like Panton-Valentine leukocidin (PVL) (David and Daum, 2010). Although, the distribution of CA-MRSA among hospital staff has been studied in detail globally, however, literature review in Saudi Arabia showed a lack of data. Therefore, the aim of this study was to study the distribution of CA-MRSA and HA-MRSA among laboratory science students and laboratory staff of a single hospital in northern region of Saudi Arabia.

MATERIALS AND METHODS

Study design and swab collection

In this study, a total of 150 subjects (125 students of laboratory science and 25 laboratory staff) of a single hospital were screened for the presence of *S. aureus*. A single non repetitive nasal swab was collected from each individual for screening. The students of first and second year were in the preparatory year and did not visit the hospital; the students of third year had just started the hospital training. Further, the fourth year students had completed one year of hospital laboratory training; whereas the fifth year students had completed two years of hospital laboratory training.

Bacterial identification

The bacterial strains were phenotypically characterized by Gram stain, catalase test, determination of tube coagulase activity and an agglutination test with a Slidex Staph Plus kit (Biomerieux). Reference strains of MRSA (NCTC 10442); methicillin sensitive *S. aureus* (MSSA) (ATCC 25923); and coagulase-negative staphylococci (CoNS) (ATCC 12228) were the control strains used. An isolated colony from each Columbia blood agar (Oxoid, UK) plate was picked, streaked onto two new Columbia blood agar plates, and incubated at 37°C for 24 h. All inocula were prepared from these subcultures. Further, the confirmation of identification of different types of *S. aureus* was carried out by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry MALDI-TOF MS (Bruker Daltonics, Germany) by direct method (Anderson et al., 2012).

Antimicrobial susceptibility testing

Five colonies were transferred into a tube containing sterile saline to prepare a suspension equivalent in density to that of a 0.5 McFarland standard. The detection of resistance was performed as per the Clinical and Laboratory Standards Institute (CLSI) 2011 guidelines, using oxacillin (1 µg) and cefoxitin (30 µg) (Oxoid, Basingstoke, UK). Further, the antibiotic susceptibility was performed by Microscan (Siemens Healthcare Diagnostics, Sacramento, CA, USA).

Molecular biology study

The confirmation of methicillin resistance among the selected bacterial isolates was confirmed by detection of *mecA* gene using polymerase chain reaction (PCR). In this method, a triplex PCR looking for *mec*A (a gene specific for methicillin resistance), *nuc* (a gene specific to detect *S. aureus*) and 16S rRNA (a genus-specific for *Staphylococcus* spp.) were used. The genes *mec*A; *nuc;* and 16s RNA were detected by the triplex PCR using methods previously described (AlKhulaifi et al., 2014). The control strains used for the three genes are NCTC 10442; ATCC 25923; and ATCC 12228, respectively.

E-mail: msm_hhscb@hotmail.com.

Table 1. The prevalence of type of *Staphylococcus aureus* among the laboratory science students and laboratory staff.

Subjects	Number of individuals	*S. aureus* (percent)	MRSA from total *S. aureus* (percent)	CA-MRSA from total MRSA (percent)	HA-MRSA from total MRSA (percent)
1st year	25	2 (8)	2 (100)	2 (100)	0 (0)
2nd year	25	3 (12)	2 (67)	2 (100)	0 (0)
3rd year	25	3 (12)	2 (67)	2 (100)	0 (0)
4th year	25	6 (24)	3 (50)	2 (67)	1 (33)
5th year	25	13 (52)	7 (54)	3 (43)	4 (57)
Staff	25	16 (64)	10 (63)	4 (40)	6 (60)
Total	150	43 (29)	26 (60)	15 (58)	11 (42)

MRSA, methicillin resistant *Staphylococcus aureus*; CA-MRSA, community-associated MRSA; HA-MRSA, healthcare-associated MRSA.

SCC*mec* typing

In this method, a multiplex PCR was used to detect the structural variations in the *mecA* element (Oliveira and de Lencastre, 2002).

Detection of Panton-Valentine leukocidin (PVL) and Toxic Shock Syndrome Toxin 1 (TSST1)

PVL and TSST1 toxins were detected by using PCR methods previously described in the literature (Becker et al., 1998; Sharma et al., 2000). The control strains used for the two genes were NCTC 13300 and NCTC 11693, respectively.

Pulsed field gel electrophoresis (PFGE)

PFGE was performed for 26 total MRSA isolates obtained in the study. It was performed according to Kaufmann method (Murchan et al., 2003), using *Sma*I-digested fragments of bacterial chromosomal DNA, with fragment separation achieved in 0.8% agarose. Electrophoresis conditions comprised using a constant voltage of 6 V/cm at 14°C and pulse times of 3.5-25 s increased linearly over 12 h (block 1), followed by 1-5 s increase over 8 h. Gel patterns were analyzed using BioNumerics software (Applied Maths) with the band tolerance set at 1.0%.

RESULTS

The results of the prevalence of *S. aureus* in this study are shown in the Table 1. Of the total nasal swabs collected, *S. aureus* were isolated from 52% laboratory science students and 64% from laboratory staff. The CA-MRSA was 100% among 1st; 2nd; and 3rd year laboratory science students and 40% among laboratory staff. The HA-MRSA were higher among laboratory staff (60%) and 4th and 5th year laboratory science students (57 and 60%, respectively).

Antimicrobial susceptibility

The results of antimicrobial susceptibility (Figure 1) showed that among CA-MRSA, 6.6% (1/15); 26 percent (4/15); 20% (3/15) and 60% (9/15) were found to be resistant to augmentin (amoxicillin and clavulanate); gentamicin; amikacin and tetracycline, respectively. Additionally, 100% (15/15) CA-MRSA were found to be susceptible to ciprofloxacin. Among the HA-MRSA, 100% (11/11) isolates were found to be resistant to all the six β-lactam antibiotics and tetracycline. Furthermore, 9% (1/11); 9% (1/11) and 45% (5/11) of HA-MRSA were found to be resistant to gentamicin; amikacin and ciprofloxacin, respectively.

Molecular biology study

The triplex PCR looking for *mecA*, *nuc* and 16S rRNA correlated very well with the phenotypic tests carried out.
Overall, 100 (15/15) and 20% (3/15) CA-MRSA isolates were found to be positive for PVL and TSST-1, respectively. The results of PVL and TSST-1 toxins detected using PCR methods are shown in Figure 1.

PFGE and SCC*mec* typing

The results of PFGE (Figure 1) showed that at 60% cut off there were two major groups. Out of 26 isolates, 15 were found to be one group consisting of all CA-MRSA with SCC*mec* type IV. The other group, 11 isolates were HA-MRSA with SSC*mec* type III and IIIA.

DISCUSSION

In hospitalized patients, MRSA has been a problem since the 1960s (Macal et al., 2014). *S. aureus* is a permanent colonizer in the anterior nares of about 20 to 30% of the general population. In comparison with general population, hospital workers are more likely to be colonized, most likely because of increased exposure (Iyer et al., 2014).

In a review, a total of 26 studies on MRSA prevalence in different regions of Kingdom of Saudi Arabia (KSA) were analyzed since 2002 to 2012. The MRSA prevalence in patients of King Fahad Medical City in 2011, in Riyadh was 50.4%, within a similar order of magnitude to other hospitals in Saudi Arabia. In a hospital in the Western region of Saudi Arabia, the MRSA prevalence was 38.9%. The prevalence of CA-MRSA in a

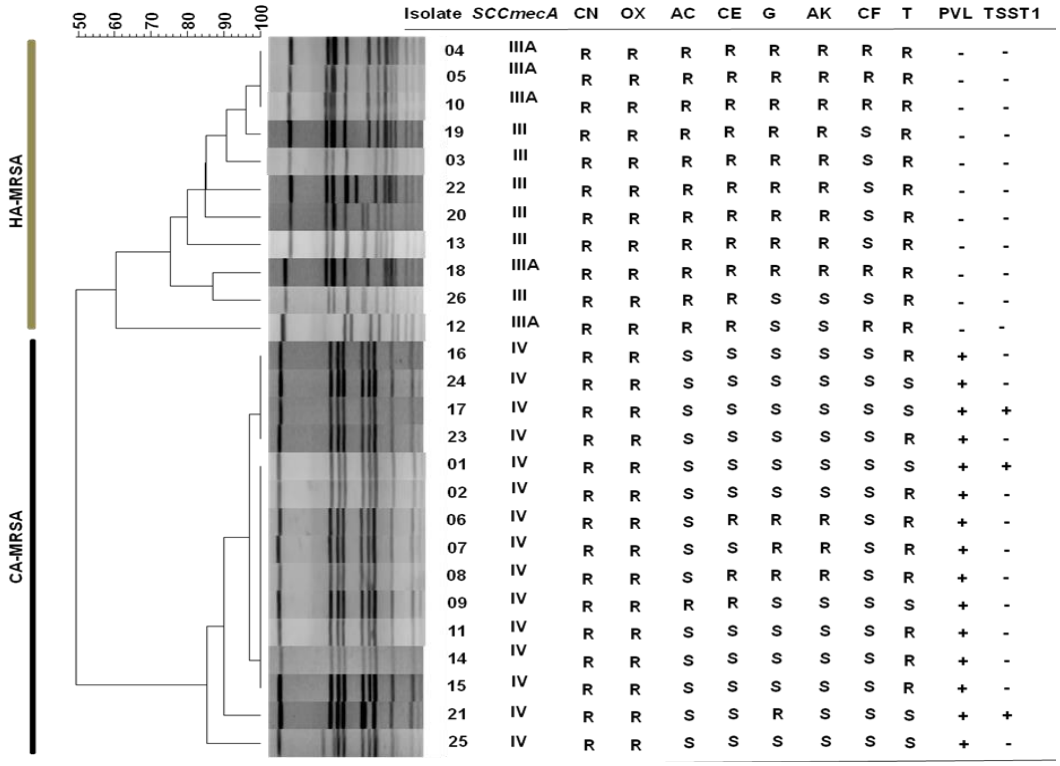

Figure 1. PFGE patterns obtained from 26 methicillin resistant *S. aureus* (MRSA) isolates cultured from students of laboratory science and laboratory staff of a hospital in north Saudi Arabia. Cluster analysis was performed using the method of DICE with UPGMA with band tolerances set to 1.0%. *SSCmecA, Staphylococcal* chromosomal cassette *mec* PCR; CN, cefoxitin; OX, oxacillin; AC, amoxicillin-clavulanate; CE, cefotaxime; G, gentamicin; AK, amikacin; CF, ciprofloxacin; T, tetracycline; PVL, Panton-Valentine Leukocidin PCR; TSST1, Toxic shock syndrome 1 PCR; R, resistant; S, susceptible; HA-MRSA, healthcare-associated MRSA; CA-MRSA, community-associated MRSA.

hospital in the Eastern region of KSA increased by six-fold during a 5-year period, between 2000 and 2008 (Monecke et al., 2012). The overall estimation of MRSA prevalence in Saudi was 35.6%, whereas MRSA prevalence mean was different between regions. While, variation in MRSA proportion exists in several cities (5.97 to 94%).

In regional perspective, Saudi has a higher prevalence of MRSA than Bahrain, Kuwait and Lebanon countries. In comparison, MRSA prevalence in Egypt, Oman, Iran and Jordan was reported to be more than 50%. Considering the worldwide scenario, the mean incidence of MRSA across China was over 50%; in Shanghai over 80% and in Spain, the prevalence of MRSA was 29.2% (Yousef et al., 2013).

In a recent report, 76% of the healthcare workers were tested positive for nasal carriage of MRSA, though they were asymptomatic. This indicates a very high incidence of MRSA (Iyer et al., 2014). Both CA-MRSA and HA-MRSA are resistant to methicillin (and all β-lactam antibiotics), however main differences exist in epidemiology, microbiologic characteristics, clinical aspects of infection, and management strategies between the two (Bukharie, 2010). Over the past decade, relatively a higher number of studies of the emergence of CA-MRSA have been published worldwide. It becomes imperative to study the distribution of MRSA among the healthcare workers, because these workers are part of community and are exposed to the hospital environment regularly (Iyer et al., 2014).

PVL-positive, community associated strains have been reported in Kuwait, Abu Dhabi, Lebanon, Egypt, Tunisia, Algeria as well as in people travelling from and to various Middle Eastern countries (Monecke et al., 2012). In our study, the percentage of CA-MRSA was found to be higher among the students (first, second and third year) with low or no exposure to hospital settings and the HA-MRSA was found to be higher among the healthcare workers (fourth year, fifth year students and hospital staff) with high exposure to hospital settings. The results of typing revealed two major lineages with one lineage (isolates 16 - 25) were being associated with PVL positivity and the carriage of SCC*mecA* Type IV (CA-MRSA). In general, this lineage is also less genetically diverse than members of lineage 2 (isolates 04 - 12). The CA-MRSA (lineage 1) isolates show a higher susceptibility

pattern to non-β-lactam antibiotics and HA-MRSA (lineage 2) isolates showed higher resistance pattern than lineage 1 isolates, which is in accordance with previously published results (Portillo et al., 2013).

As the epidemiology of MRSA disease changes, including both community- and health care-associated disease, accurate information on the scope and magnitude of the burden of MRSA disease in the Saudi population is needed to be studied in detail for infection prevention and control. As per our knowledge, this is the first study of the incidence and distribution of CA-MRSA conducted among the laboratory science students and laboratory staff of a hospital in North Saudi Arabia. Our study reports a high percentage of CA-MRSA isolates among the healthcare workers who have lesser exposure to the hospital environment as compared to those with high exposure. Also, the genetic relatedness, presence of PVL and identical antibiogram of our CA-MRSA makes this study interesting as carriage of these isolates in the healthcare workers may play a key role in the epidemiology and pathogenesis of infection in the hospitals in future. Strategies to interrupt transmission of CA-MRSA to hospitalized patients via healthcare workers like regular hand washing and use of antiseptics should be implemented. Continuing surveillance is needed more accurately to assess the prevalence, geographic distribution and epidemiology of community acquired MRSA at broader level.

Conflict of interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Characterization and identification of lactic acid bacteria isolated from traditional cheese (*Klila*) prepared from cow's milk

Guetouache, M.[1,2]* and Guessas, B.[2]

[1]Department of Microbiology and Biochemistry, Faculty of Science, University of Mohamed Bouadiaf M'sila, 28 000, Algeria.
[2]Laboratory of Applied Microbiology, Department of Biology, Faculty of Sciences, Oran University. Algeria.

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Various types of fermented dairy products exist worldwide. Their nature depends on the type of milk used, pretreatment, fermentation conditions and subsequent treatment. The fermentation of milk primarily involves lactic acid bacteria (LAB). Among these, the *Klila* is a hard variety cheese made by using the traditional procedures in the home, without using a starter culture. The different samples of traditional cheese (*Klila*) studied were collected from the rural area of the province of Djelfa. Isolates were phenotypically characterized by their capability to ferment different carbohydrates and additional biochemical tests. 132 lactic acid bacterial strains were isolated, purified and identified to all belong to the genus, *Lactobacillus,* their proportion were *Lactobacillus plantarum* (18.94%), *Lactobacillus casei* (18.18%), *Lactobacillus fermentum* (21.97%), *Lactobacillus acidophilus* (12.88%), *Lactobacillus brevis* (14.39%), *Lactobacillus alimentarus* (03.03%), *Lactobacillus intestinalis* (06.06%) and *Lactobacillus helveticus* (04.56%). These lactic acid bacteria were isolated against *Staphylococcus aureus*. Isolates *L. fermentum, L. intestinalis* and *L. acidophilus* were selected for their strong bactericidal activity against *S. aureus*.

Key words: *Klila*, lactic acid bacteria, identification, characteristics, *Lactobacillus, Staphylococcus aureus*.

INTRODUCTION

Milk is the lacteal secretion obtained by the complete milking of mammals. Due to its high nutritional value for human beings, it is a significant food of nutrition for immense population on earth. When temperature is suitable for growth of microorganisms, the milk appears as an excellent medium for their growth. The milk is contaminated very easily if it is handled carelessly and produced unhygienic-cally, resulting in its early spoilage (Bahanullah et al., 2013). Fermented milk is a dairy product providing the human diet with nutritious compounds of varied flavors, aromas and textures. These products are based on the metabolic activity of lactic acid bacteria which ferment sugars, especially glucose and galactose, to produce lactic acid and aroma substances that give typical flavors and tastes to fermented products. Several types of fermented milk products have been reported to exist through-out the world. The most popular ones in North African are *Jben, Lben, Klila* and *Raib* (Mechai and Kiran, 2008). The name

*Corresponding author. E-mail: mouradeg33@yahoo.com. Tel: 213 0549537624.

"cheese" is reserved to fermented product or not obtained by coagulating milk, cream, skim milk, or a mixture of them, followed by draining. The cheese is made either by the traditional method in the rural environment and traditionally or by the semi-industrial or industrial methods which remains limited (Rhiat et al., 2013).

In Algeria, many traditional dairy products are not identified and studied; several types of cheeses are classified and identified in different parts of our country. Among these different types, we mentioned the following names *Mechouna* cheeses, *Bouhezza*, *Madeghissa*, *Klila, Djben Takammerite*, *Aoules*, *Igounanes* and *Takammerite*. In a variety of ecological niches, micro-organisms compete with each other for survival and through evolution from unique flora. In some food ecosystems, lactic acid bacteria constitute the dominant microbiota of these bacteria widely distributed in the nature and occurring naturally as indigenous micro-biota in raw milk as Gram-positive bacteria that play an important role in many foods and feed fermentations. These organisms are able to produce antimicrobial com-pounds against competing microbiota, including food-borne spoilage and pathogenic bacteria. In this group are included representatives of the genus *Lactobacillus, Enterococcus, Bifidobacterium, Aerococcus, Leuconostoc, Pediococcus, Streptococcus, Carnobacterium, Oenococcus, Weissella, Tetragenococcus, Vagococcus* and *Lactococcus* (Dortu and Thonart, 2009). The lactic acid fermentation, which these bacteria carry out, has long been known and applied by humans for making different food-stuffs. For many centuries, lactic acid bacteria have served to provide an effective form of natural preservation. In addition, they strongly determine the flavor, texture and, frequently, the nutritional value of food and feed products. They could be isolated from soil, water, plant, silage, waste product, and also from the intestinal tract of animals and humans. Since decades, by these processes, the application of well-studied starter cultures was established. They should possess stable fermentation characteristics and should be resistant to bacteriophages (Lee, 1996). The “wild” isolate, in biotechnological aspect are perceived as bacteriocin producers and probiotics (Tserovska et al., 2002). This study was aimed at the isolation and taxonomic determination of large number of lactic acid bacteria from traditional fermented milk (*Klila)* prepared from cow's milk and characterization of different groups of microbiota, acidifying power and antimicrobial producer bacteria using classical methods.

MATERIALS AND METHODS

Samples collection

The various samples of dry *Klila* were processed by traditional procedure in the home. The different samples of traditional cheese (*Klila*) studied were collected from the rural area of the province of Djelfa. They were transported to the laboratory under refrigeration (4°C) and analyzed immediately; the pH measurement of the samples (sample preparation was carried out by dissolving 5 g of *Klila* in 25 ml of distilled water with a neutral pH) was performed by a pH meter with an Orion Research type combination electrode and previously calibrated with buffer solutions at pH 4 and 7. 10 ml of sample was transferred to a small beaker and 5 drops of phenol-phthalein was added to 1% indicator. The sample was titrated with 0.1 N NaOH. Note that the sample should be just barely pink (Rhiat et al., 2013).

Microbiological analysis

Microbiological analysis was performed for controlled traditional *Klila* to search: total aerobic mesophilic flora (FAMT) enumerated on PCA agar (plate count agar), incubated for 24 h at 30°C. The search for total coliforms wassought on désoxycolate citrate agar (DCL) incubated for 24 h at 37°C for total coliforms and at 44°C for fecal coliforms, fecal streptococci were counted on sodium azide after incubation for 48 h at 37°C. Staphylococci were counted on Chapman medium containing a high concentration of NaCl (75%) tolerated only by staphylococci, incubation at 37°C for 24 to 48 h (Labioui et al., 2009; Bouzaid et al., 2012), for Salmonella, a pre-enrichment on selenite-cysteine medium was provided for 12 h at 37°C, followed by an enrichment on bouillon of tetrathionate for 24 h at 37°C, then the enumeration and isolation were carried out on SS medium (*Salmonella*-*Shigella*) after 24 h of incubation at 37°C and the sulphitoreductor-clostridia were counted in the culture medium reinforced *Clostridium* agar in tubes to promote anaerobic conditions, with thermic treatment for 10 min at 80°C to activate the spores of clostridia: they can persist in a latent form in milk, germinate as soon as conditions are favorable and secrete toxic substances. The tubes are incubated for 48 h at 37°C. Only black colonies are counted. The microbiological analysis is performed in three steps: preparation of dilutions, seeding in the culture medium and enumeration of microorganisms. A count of lactic acid bacteria was responsible for the fermentation and acidification of milk, they were counted on the MRS agar (De Man et al., 1960) and incubated for 48 h at 30°C and the counts of yeasts and moulds were determined using potato dextrose agar (PDA), acidified with 10% tartaric acid to pH 3.5 by incubating at 30°C for 3-5 days.

Study of lactic microfloras

Isolation and purification of lactic acid bacteria

Each 25 g sample was aseptically weighed and homogenized by adding 225 ml of physiological saline solution for first dilution (10^{-1}). Next dilutions (10^{-2} to 10^{-7}) were made in 0.85% sterile saline. For lactic acid bacteria (LAB) isolation, 1 ml of the appropriate dilutions was plated on MRS and GM17 agar medium, respectively. The plates were incubated at 30 and 45°C for 72 h under aerobic and anaerobic conditions. Further decimal dilutions were prepared from this homogenized mixture (Kivanç et al., 2011; Terzic et al., 2014). The 0.1 ml from each dilution was then sub cultured, in duplicate, into the M17 and MRS agars used for isolating lactic acid bacteria. To prevent the growing of yeasts, the media were then supplemented with 100 mg/l of cycloheximide before being incubated at the appropriate temperatures for 2-3 days. The MRS agar plates were incubated anaerobically at 42, 35 and 30°C for three days, in order to provide an optimal temperature for growing thermophilic lactobacilli, mesophilic lactobacilli and Leuconostoc, respectively. M17 agar plates were also incubated aerobically at 30°C for 2 days, in order to set up an optimal temperature for growing lactococci. To perform the total counts, the higher dilutions were used (Azhari, 2011). Colonies were randomly selected and streak plating was then used to purify the isolates which were subsequently kept in two different conditions including at 4°C for MRS and M17 plates

and at -20°C for M17 and MRS broths supplemented by 20% glycerol for further use (Mathara et al., 2004). All isolates were examined for Gram reaction, production of catalase and oxidase activity. Gram-positive and catalase- and oxidase-negative isolates were stored for further analyses. Purification of the isolates was done by repeated pour plating technique using the same agar medium until pure cultures were obtained. Pure cultures were transferred and maintained on de Man Rogosa and Sharpe (MRS) agar stabs. Duplicate tubes of the isolates were prepared, one tube was stored in refrigerator as stock culture, and the other tube was used for identification studies (Neti and Erlinda, 2011).

Identification of lactic acid bacteria isolates

Isolates were identified using the following tests: ammonia production from arginine, CO_2 production from glucose, and growth at different temperatures (10, 15, 30, 37 and 45°C), growth at different pH values, and growth at different NaCl concentrations (Schillinger and Lucke, 1989). Each strain under examination was subcultured twice overnight in MRS broth. All strains were initially tested for Gram reaction, catalase production and spore formation (Harrigan and McCance, 1976). Cell morphology and colony characteristics on MRS agar were also examined, and a separation into phenotypic groups was undertaken. Only the Gram-positive, catalase-negative isolates were further identified. Growth at different temperatures was observed in MRS broth after incubation for 5 days at 15, 37 and 45°C. Hydrolysis of arginine was tested in M16BPC (Thomas, 1973). Growth in the presence of 4 and 6.5% NaCl performed in MRS broth for 5 days. Utilization of citrate was realized in Kempler and Mc Kay (1980) medium. Production of acetone from glucose was determined using Voges-Proskauer test (Samelis et al., 1994). To perform the biochemical tests, an MRS-BCP broth medium (BCP 0.17 g/l) was used. The carbon source was added to the sterile basal medium as filter sterilized solution to a final concentration of 1%. Carbohydrates utilization was assessed at the 24th and 48th h. All strains were tested for fermentation of the following 15 sugars: L-Arabinose, ribose, D-xylose, mannitol, sorbitol, cellobiose, maltose, lactose, melibiose, trehalose, mannose, rhamnose, esculine, sucrose and D-raffinose. To ensure anaerobic conditions, two drops of sterile liquid paraffine were placed in each tube after inoculation.

Kinetic of pH and acidity lactic acid production

The strains were initially grown on MRS broth and then in sterile reconstituted skim milk supplemented with yeast extract (0.3%) and glucose (0.2%) for two successive subcultures. Sterile reconstituted skim milk (100 ml) was inoculated with 1% of an 18 h preculture (Durlu et al., 2001) After gentle agitation, culture was divided into tube (10 ml/tube) and incubated at 30°C. At a regular interval time, samples were aseptically collected every 2 h. A volume of 1 ml culture samples was used for making suitable serial dilutions up to 10^{-8} by incorporating 1 ml into 9 ml of sterile saline water in sterile tubes. Enumeration of LAB was determined using selective media, MRS agar. The plates were incubated at 30°C for 48 h. After incubation, colonies were enumerated, recorded as colony forming units (cfu/ml). Only plates containing between 30 and 300 colonies were retained (Khedid et al., 2009). The generation time and growth rate were calculated in the exponential growth phase. The kinetics of the changes in pH and acidity were also followed by measuring pH and Dornic acidity. To measure Dornic acidity, we added 5 drops of alcoholic solution of 1% phenolphthalein as an indicator of the color change point in 10 ml of culture samples. We titrated sodium hydroxide N/9 (NaOH), until the sample changed color from white to light pink. The volume of NaOH sunk was recorded. The acidity was expressed in degrees Dornic (°D) (1°D = 0.1 g lactic acid/liter and acidity = volume of NaOH x 10) (Va´zquez et al., 2013). Titrable acidity of lactic acid was calculated according to FAO (1986).

Screening for antagonistic activity

The many methods described for the detection of isolates bacteriocin-producing lactic acid is based on the premise that these protein substances can diffuse into a solid culture medium or semi solid which was previously inoculated with a target strain (*Staphylococcus aureus* ATCC 65 38). The bacteriocin production inhibitor is detected by the power of the filtrate microorganism tested growth target seed. Isolates of lactic acid bacteria after culture on medium MRS at pH 6.8, incubation at 30°C were tested for their antibacterial activity following diffusion method agar TSA (Tryptic Soy Agar, Difco, Detroit, USA) (Barefoot and Klaenhammer, 1983). The supernatant containing the crude extract is collected by centrifugation bactériocinique adjusted to neutral pH of 6.5 to 7 with 10 M NaOH neutralizing the extract bactériocinique which eliminates the effect of organic acids. The extract was then filtered on millipore filters sterile 0.22 µ in diameter, the antimicrobial activity was determined for each selected isolate of *Lactobacillus*. Petri dishes were overlaid with 15 ml of molten agar (1%), inoculated with 30 µl of an overnight culture of the indicator microorganism, in which wells were formed. Wells, mm in diameter and of 30 µl in capacity, were formed by carving the agar with a cork borer. Afterwards, 30 µl of an overnight culture of the putative inhibitor strain were placed in each well. The plates were then incubated aerobically for 24 h at a temperature conducive for growth of the indicator microorganism and were subsequently examined for zones of inhibition. Inhibition was recorded as negative if no zone was observed around the agar well. Each antagonistic activity was related to the area (2 mm) of the inhibition zone displayed (Mathur and Singh, 2005).

RESULTS AND DISCUSSION

Physicochemical analysis

The pH range for traditional cheeses (*Klila*) was 3.8 to 4.8 with an average of 4.2. Titratable acidity of *Klila* samples varied from values as low as 68°D to values as high as 91°D. Mean titratable acidity value was 79.4°D; these values are almost similar to that reported by Rhiat et al. (Table 1).

Microbiological analysis

The coliforms and pathogenic microorganisms, *S. aureus* and *Salmonella* were not detected; Lactic acid bacteria are by far the major microbial group in traditional cheeses (*Klila*) products. The microbiological analysis showed an average of FAMT of about $2.1x10^3$, $1.5\ x10^3$, $2.6\ x10^3$, $2.1\ x\ 10^3$ and $2.8x10^3$ cfu/ml, respectively, in samples S2, S3, S4 and S5. However, the staphylococci were not detected. These values are almost similar to that found by Mennane et al. (2008). The total and fecal coliforms were found in sample S3 and S5, the levels of coliforms (total, fecal) found in two samples are lower than those reported by Hamama and EL Mouktafi (1990). We also noticed the absence of pathogenic flora especially for controlled products. But the burden of yeasts, $1.2x10^2$ to $1.2x10^2$ cfu/ml is a normal standard (Table 2).

LAB were enumerated in traditional cheeses (*Klila*) using usual media by the classic method. The presumptive lactic acid bacteria levels varied from $0.3x10^4$ to $4.2x10^4$ cfu/ml with an average of $2.2x10^3$.

Table 1. Results of physicochemical characteristics of traditional cheese (*Klila*).

Physicochemical characteristics	Sample				
	S1	**S2**	**S3**	**S4**	**S5**
pH	3.8	3.9	4.2	4.3	4.8
°D (Dornic acidity)	68	71	79	88	91

Lactic acid bacteria counts found in traditional cheeses (*Klila*) were low as compared to LAB levels already reported in other types of traditional dairy product such as Jben and cow's milk (Khedid et al., 2009; Labioui et al., 2009) (Table 2).

Study of lactic microbiota

A total of 132 isolates from traditional cheese (*Klila*) isolates were Gram-positive, catalase-negative, non spore-forming and short rod or cocobasilli shaped. These isolates were selected for identification and antagonism analysis, the results of the isolation and identification of the standard physiological and biochemical tests (Table 3) identified the isolates as 18.94% isolates of *Lactobacillus plantarum*, 18.18% isolates of *Lactobacillus casei,* 21.97% isolates of *Lactobacillus fermentum,* 12.88% isolates of *Lactobacillus acidophilus,* 14.39% isolates of *Lactobacillus brevis,* 03.03% isolates of *Lactobacillus alimentarus,* 06.06% isolates of *Lactobacillus intestinalis* and 04.56% isolates of *Lactobacillus helveticus.* We have divided the Lactobacilli group into three subgroups according to Orla-jensen (1919) and Moreik (2011) as follows: *L. plantarum, L. alimentarus* and *L. casei* subsp. *casei* are mesophilic facultative hetero-fermentative, *Lactobacillus helveticus, L. acidophilus, L. intestinalis* and *L. fermentum* which are thermophilic obligate homo-fermentative and *L. brevis* which are mesophilic obligate hetero-fermentative (Azadnia and Khan, 2009).

The morphological, biochemical and physiological characterization of the isolates revealed that all the isolates that produced highest lactic acid among each group are *L. acidophilus, L. fermentum* and *L. plantarium.* All isolates fermented the same carbohydrates; Olarte et al. (2000) noted that the presence of *L. plantarum* in the cheese (Cameros) from goat's milk decreased the number of the enterbacteria and fecal coliforms in the final product (Table 3).

Kinetics of acidification and growth evolution

The variation of acidification was monitored for all isolates, as shown in Figure 1. The diminution of pH of the milk is due to the production of lactic acids from lactose fermentation (Thomson et al., 1994). The amount of lactic acid varies according the isolates and their capacity and the rate of degradation of the lactose. Thus, according to their ability of acidification, the strains were divided as follows: highly acidifying isolates (include GM91 and GM14) that coagulate milk before 18 h of incubation, low acidifying isolates (strains GM33 and GM88) that coagulate milk after 18 h of incubation and the remaining isolates coagulate milk after 18 to 24 h of incubation.

The initial pH of skim milk was 6.2 to 6.4 for all the tested isolates. Then, the pH decreases with time to reach 3-3.4 in highly proteolyses isolates. Regarding the acidity, we noteed that after 2 h of incubation, the amount of lactic acid was measured (15-22°D) for all our isolates. The acidity increases with the time in a variable way to arrive until 74°D after 24 h with isolate GM14 and up 31°D with the isolate GM11. The acidity produced can reach 63 and 74°D for thermophilic and mesophilic isolates, respectively.

Antibacterial activity

The antimicrobial activity of *Klila* isolated from lactic acid bacteria were detected using the method of well diffusion test on the basis of their ability to inhibit the growth of the indicator isolate *S. aureus* ATCC 65 38. Based on the results, a total of 5 different traditional *Klila* samples analyzed. 09 bacteria inhibitor of production, which are alleged to constitute lactic acid bacteria were isolated (Table 4).

Five of these isolates producing inhibitor were selected for further study on the basis of their relatively broad antimicrobial spectrum (Figure 2). The sensitivity of the antibacterial substances produced by lactic acid bacteria in α-chymotrypsin, pepsin, catalase, and the lipase was determined in reproducible and controlled conditions indicated in Table 5.

Inhibitor compounds produced by strains inhibitors showed different patterns of sensitivity. All were completely inactivated by α-chymotrypsin alone which was resistant to pepsin (isolate GM11), whereas the compounds produced by GM91 and GM14 isolates were inactivated after treatment with the lipase, indicating that these substances can have inhibitory lipid moiety in their chemical composition. The inhibitory compounds produced by the three isolates showed great resilience to thermal treatments.

In another way, bacteriocin has proved stable over a wide pH range with all peptides, now some antimicrobial activity in the pH range from pH 4-7. According to Allouche et al. (2010), recent bacteriocin is very sensitive to pH. Its stability was detected at a pH range of 3.5 to 6.5. In this study, bacteriocin produced by isolates GM91 and GM14 had the same profile and were active at pH values 4-6 (Table 5). In a similar study, the work of Zamfir et al. (1999) reported that the bacteriocin produced by *L. acidophilus* develop a positive activity against *S. aureus*.

Table 2. Results of microbiological analysis (cfu/ml) of traditional cheeses (*Klila*).

Microbiological analysis	Sample					
	S1	S2	S3	S4	S5	M
Yeast 10^2	1.2	1.5	2.0	2.2	1.3	1.64
Total aerobic mesophilic 10^3	2.1	1.5	2.6	2.1	2.8	2.22
Total coliforms 10^3	0.0	0.0	2.0	0.0	2.5	2.25
Fecal coliforms 10^3	0.0	0.0	1.2	0.0	2.5	1.85
Staphylococci 10^3	0.0	0.0	0.0	0.0	0.0	0.00
Lactic microflora 10^4	2.1	3.5	0.8	4.2	0.3	2.18

Table 3. Morphological, cultural, physiological characteristics of isolated isolates.

Species	GM14	GM91	GM62	GM12	GM33	GM88	GM67	GM11
Gas from glucose	+	+	+	-	-	-	+	+
Motility	-	-	-	-	-	-	-	-
Hydrolysis of								
ADH	-	+	+	-	-	-	-	-
Citrate	+	+	-	+	-	-	+	+
Growth at different temperature (°C)								
15	-	-	+	+	+	-	+	-
30	+	+	+	+	+	+	+	+
45	+	+	-	-	-	+	+	+
Growth at different pH								
6.5	+	-	-	+	+	-	+	-
9.6	-	-	-	-	-	-	-	-
Growth in the presence of NaCl								
4%	+	-	-	+	+	+	+	+
6.5	+	+	+	+	+	+	+	+
9.6	-	-	-	-	+	-	-	-
Sugar fermentation								
Arabinose	+	+	+	-	-	-	-	-
Cellobiose	+	+	-	+	+	-	+	-
Mannitol	-	-	-	+	-	-	+	+
Mannose	+	+	-	+	+	+	+	-
Melebiose	+	+	+	+	-	-	-	+
Raffinose	+	+	+	+	-	-	-	+
Ribose	-	+	+	+	+	-	+	-
Lactose	+	+	+	+	+	+	-	+
Rhamnose	-	-	-	+	-	-	-	-
Sorbitol	-	+	-	+	+	+	-	+
Xylose	-	-	+	-	-	-	-	-
Tehalose	-	-	-	+	+	+	+	-
Maltose	+	+	+	+	+	-	+	+
Esculine	-	-	+	+	+	+	+	-
Sucrose	+	+	-	+	+	-	-	+

GM12: *Lactobacillus plantarium*, GM33: *Lactobacillus alimentarus*, GM67: *Lactobacillus casei* subsp. *casei,* GM88: *Lactobacillus helveticus*, GM14: *Lactobacillus acidophilus*, GM11: *Lactobacillus intestinalis*, GM91: *Lactobacillus fermentum*, GM62: *Lactobacillus brevis.*

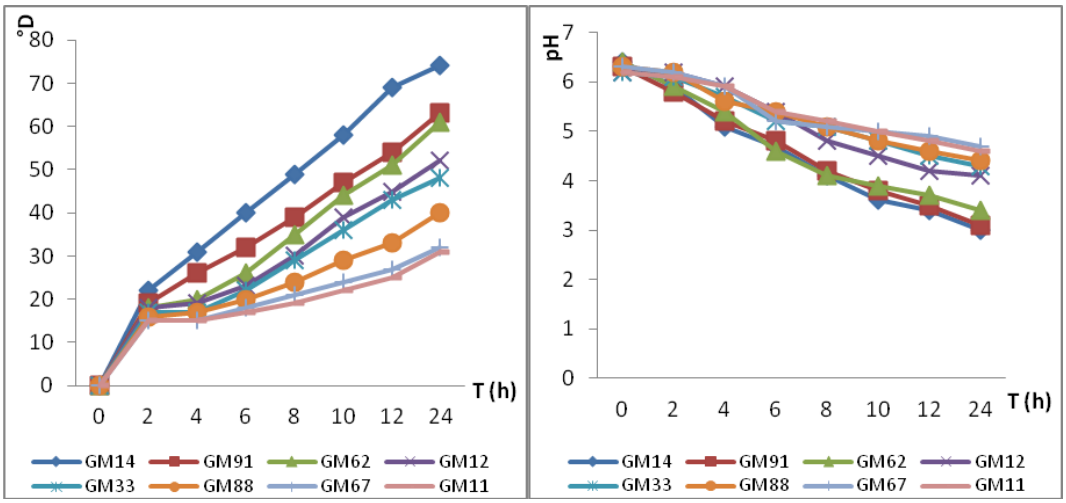

Figure 1. Kinetics of degree dornic acidity of the different isolates in milk medium.

Table 4. Antagonism of *Staphylococcus aureus* ATCC 65 38 by *Lactobacillus* isolates using agar diffusion method.

Statistical analysis	Isolates test								
	GM14	GM91	GM62	GM11	GM12	GM67	GM60	GM03	GM46
Mean	9.950	9.150	9.275	6.800	6.050	3.825	2.575	2.850	2.425
SD	0.9883	0.3109	0.2630	0.4830	0.2380	0.4500	0.4113	0.5066	0.4573
SE	0.9883	0.3109	0.2630	0.4830	0.2380	0.4500	0.4113	0.5066	0.4573

SD: Standard deviation; SE: standard error.

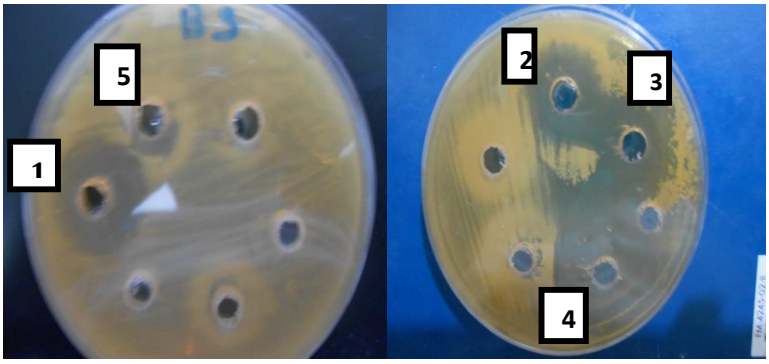

Figure 2. Inhibition of *S. aureus* ATCC 65 38 by the cell-free supernatants of the five producing isolates using the agar well-diffusion assay: 1: GM11, 2: GM62, 3: GM91, 4: GM14 and 5: GM12.

Table 5. Action of proteolytic enzymes, pH and heat treatment on the antimicrobial activity of crude extracts against the growth of *Staphylococcus aureus* ATCC 65 38.

Crude extracts	Enzymes			pH			Heat treatment °C/20 min	
	α-Chymotrypsin	Lipase	Pepsin	3	5	7	100	120
GM14	-	-	-	+	+	+	+	+
GM91	-	-	-	+	+	+	+	+
GM62	-	-	-	-	+	+	+	-
GM11	-	-	+	-	+	+	-	-
GM12	-	+	-	-	-	+	-	-

Conflict of interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

β-Lactam - β-lactamase inhibitor combinations as the choice therapy for multidrug resistant Acinetobacter

Farhan Rasheed*, Raja Kamran Afzal and Muhammad Jan Leghari

Microbiology Department, Combined Military Hospital, Lahore, Pakistan.

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Acinetobacter, an important nosocomial pathogen, is capable of causing infectious outbreaks in critically ill patients which results into high morbidity and mortality worldwide. It is rated among top seven pathogens that disturb the health care delivery system. The situation has become complicated due to the organism's capability to acquire diverse resistance mechanisms. This has resulted in the emergence of multidrug resistant and pan-drug resistant strains. A total of 100 clinical isolates of *Acinetobacter* spp. were evaluated against five β-lactam – β-lactamase inhibitor combinations by modified Kirby Bauer disc diffusion method using Mueller-Hinton agar. Zone sizes were interpreted according to CLSI 2012 guidelines. Out of 100 isolates, 85 were *Acinetobacter baumannii*, 9 were *Acinetobacter johnsonii* and 6 were *Acinetobacter lwoffii.* Eighty four isolates of *A. baumannii,* 8 isolates of *A. johnsonii* and all 6 isolates of *A. lwoffii* were multidrug resistant. One isolate from each of *A. baumanni* and *A. johnsonii,* and no isolate of *A. lwoffii* were susceptible to co-amoxiclav. Twenty eight isolates of *A. baumanni,* one isolate of *A. johnsonii* and no isolate of *A. lwoffii* were susceptible to ampicillin-sulbactam. Forty one (41) isolates of *A. baumanni,* one isolate of *A. johnsonii* and no isolate of *A. lwoffii* were susceptible to piperacillin-sulbactam. Eight isolates of *A. baumanni,* one isolate of *A. johnsonii* and no isolate of *A. lwoffii* were susceptible to piperacillin-tazobactam. Forty eight isolates of *A. baumannii,* one isolate of *A. johnsonii*, and no isolate of *A. lwoffii* were susceptible to cefoperazone-sulbactam. Cefoperazone-sulbactam was the most effective combination against 49% isolates of Acinetobacter. Ninety one percent isolates were resistant to piperacillin-tazobactam. Combinations having sulbactam were more effective as compared to others. This work also support the postulate that sulbactam, though not an antimicrobial, but does possess antibacterial activity against Acinetobacter species.

Key words: Acinetobacter, β-lactam – β-lactamase inhibitor combinations, cefoperazone-sulbactam.

INTRODUCTION

Acinetobacter is a Gram negative cocco-bacillus, aerobic, pleomorphic, non-fermenting, non-fastidious, non-motile, catalase-positive and oxidase-negative opportunistic pathogen. This genus consists of 35 species (Turton et al., 2005). Out of these, *Acinetobacter baumannii* is responsible for about 80% of clinical conditions (Sebeny et al., 2008). Acinetobacter has a high incidence among immunocompromised individuals, particularly those who

*Corresponding author. E-mail: dr.farhanrasheed@gmail.com.

have experienced a prolonged hospital stay (Montefour et al., 2008). It has been observed to colonize the skin as well as the respiratory and oropharyngeal secretions of hospitalized patients (Sebeny et al., 2008). Propensity to tolerate drying and resistance to multiple classes of antibiotics are the other key factors that enable this organism to survive and spread in the hospital environment. Bacteremia, urinary tract infections, pneumonia, and meningitis, are the main complications resulting from *Acinetobacter* spp. induced nosocomial infections (Ionescu and Constantiniu, 2004). A*. baumannii* earlier became one of the most common hospital acquired pneumonia causing pathogen (Glew et al., 1977). There are some reports documenting *A. baumannii* the cause of community-acquired pneumonia also (Leung et al., 2006). A study in USA showed that, almost 4% of combat wound infections in battle field soldiers were due to *Acinetobacter* spp. (CDC, 2002). The ability of *A. baumannii* to form biofilms allows it to grow in unfavorable conditions and environments also. *A. baumannii* has been shown to form biofilms on inanimate surfaces, which can include glass and equipment used in intensive care units, and on biotic surfaces such as epithelial cells (Gaddy and Actis, 2009).

The increasing bacterial resistance to carbapenems or even to colistin or tigecycline is of great concern because these antibiotics are the last therapeutic regimen for many bacterial infections (Hoffmann et al., 2010; Peleg et al., 2008; Dijkshoorn et al., 2007; Bergogne-Berezin and Towner, 1996). Bacterial strains are referred to as multi-drug resistant, when resistance to three or more classes of antibiotics is demonstrated (Peleg et al., 2008). The emergence of resistance to all β-lactams especially the broad spectrum carbapenems depicts the capability of *A. baumannii* strains to change their response rapidly to environmental changes by selective pressure. Acquiring resistance mechanism due to chromosomal reassortment and through plasmids have made *A. baumannii* a pathogen of emerging threat. Although, we have limited data of genetic reassortment of *A. baumannii* and other species of Acinetobacter, especially *A. baylyi*, these pathogens are highly competent in acquiring resistance (Bacher et al., 2006; Vaneechoutte et al., 2006).

Acinetobacter can acquire resistance either by enzymatic method or non-enzymatic methods. Mostly, *A. baumannii* acquire resistance to β-lactams by producing β-lactamases, in particular to β-lactams during enzymatic degradation by β-lactamases (Bou et al., 2000; Tsakris et al., 2006). The enzymatic modification is another tool for resistance that is genes coding for aminoglycoside modifying enzymes are present in multidrug-resistant *A. baumannii* strains (Lee et al., 2005; Zarrilli et al., 2004).

In resistance mechanisms of Acinetobacter, all of the major enzyme classes have been found, including acetyltransferases, nucleotidyltransferases, and phosphotransferases (Hujer et al., 2006; Nemec et al., 2004). The resistance to β-lactams, including carbapenem, has also been associated with non-enzymatic resistance mechanisms, including changes in outer membrane proteins (OMPs) (Gribun et al., 2003; Mussi et al., 2005), multidrug efflux pumps (Heritier et al., 2005; Higgins et al., 2004), and alterations in the affinity of penicillin-binding proteins (Siroy et al., 2006). The resistance to tetracycline group may be mediated by efflux or ribosomal protection (Fluit et al., 2005). The term “pan-resistance” has been used to describe strains of Acinetobacter species that are resistant to all standard antimicrobial agents tested except colistin (Paterson, 2006).

The broad spectrum of activity of β-lactamase inhibitors in combination with β-lactam antibiotics originates from the ability of respective inhibitors to inactivate a wide range of β-lactamases produced by Gram positive, Gram negative and even acid-fast pathogens. Clinical experience confirms their effectiveness in the empirical treatment of respiratory, intra-abdominal, skin, and soft tissue infections. Their role in treating various multidrug resistant pathogens is gaining importance (Perez-Llarena and Bou, 2009). The aim of the present study was to test the effectiveness of 5 different combinations of β-lactam- β-lactamase inhibitors against multi drug resistant clinical isolate of *Acinetobacter* spp.

MATERIALS AND METHODS

This descriptive, cross-sectional study was carried out in the Department of Microbiology, Combined Military Hospital, Lahore, from January to October 2012. Clinical specimens like blood, pus, double lumen tip, ascitic fluid, tracheal aspirate, naso-bronchial lavage (NBL), cerebrospinal fluid (CSF), high vaginal swab (HVS) were cultured on blood and MacConkey agar, while the urine samples on were cultured on cysteine lactose electrolyte deficient (CLED) agar. Later the isolates were identified by Gram staining, a positive catalase test and negative cytochrome oxidase test. Species level identification was done by API-20NE (biomerieux, France). Duplicate samples of the same patient during the same episode of illness were excluded. A total of 100 clinical isolates of *Acinetobacter* spp. were included in this study. Antimicrobial susceptibility testing of the isolates was carried out using the modified Kirby-Bauer disc diffusion method. Bacterial suspensions equivalent to 0.5 McFarland turbidity standard were prepared and inoculated on Mueller Hinton agar plates. Isolates resistant to three or more classes of antibiotics (aminoglycoside, quinolones and third generation cephalosporin) were labelled as multidrug resistant. Antibiotic discs of co-amoxiclav 30 µg (amoxicillin 20 µg + clavulanate 10 µg), ampicillin-sulbactam 20 µg (ampicillin 10 µg + sulbactam 10µg), piperacillin-tazobactam 110 µg (piperacillin 100 µg + tazobactam 10 µg), piperacillin-sulbactam 130 µg (piperacillin 100 µg + sulbactam 30 µg), cefoperazone-sulbactam 105 µg (cefoperazone 70 µg + sulbactam 35 µg), (Oxoid, UK) were applied followed by incubation at 35°C for 18 - 24 h. The results were interpreted following the Clinical and Laboratory Standards Institute guidelines 2012 (CLSI, 2012) as shown in Table 1.

American Type Culture Collection (ATCC) *Escherichia coli* 35218 was used as the quality control strain. Data was analyzed using Statistical Package for Social Sciences (SPSS) version 19. Qualitative variables for example clinical specimens and antimicrobial susceptibility were expressed as frequency and percentages.

Table 1. Clinical and Laboratory Standards Institute guidelines 2012.

β-Lactam-B-lactamase Inhibitor combinations drugs	Sensitive (zone size in mm)	Intermediate (zone size in mm)	Resistant (zone size in mm)
Amoxicillin-clavulanate (30 µg)	≥ 18	14-17	≤ 13
Ampicillin-sulbactam (20 µg)	≥ 15	12-14	≤ 11
Piperacillin-tazobactam (110 µg)	≥ 21	18-20	≤ 17
Piperacillin-sulbactam (130 µg)	≥ 21	18-20	≤ 17
Cefaperazone-sulbactam (105 µg)	≥ 21	18-20	≤ 17

Table 2. Percentage of MDR Acinetobacter.

Isolates	MDR Acinetobacter
Acinetobacter baumannii	84 (98.8%)
Acinetobacter johnsonii	8 (88.8%)
Acinetobacter lwoffii	6 (100%)
Total	98 (98%)

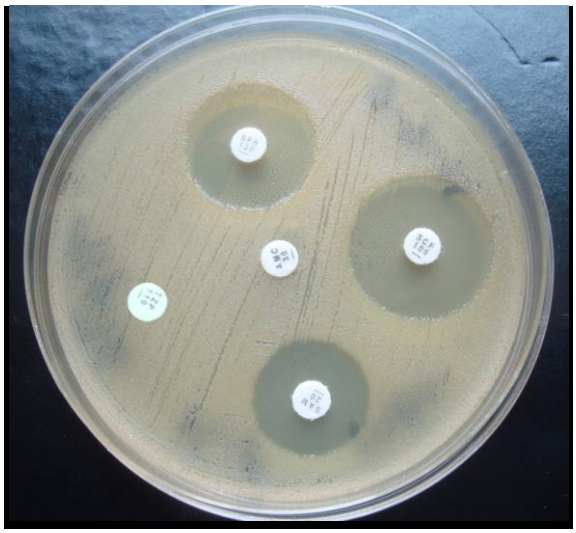

Figure 1. Susceptibility of Acinetobacter against β-lactam - β-lactamase Inhibitors.

RESULTS

Out of 100 isolates, 85 were *A. baumannii*, 9 were *A. johnsonii* and 6 were A*. lwoffii.* Out of 85 isolates of *A. baumannii,* 84 (98.8%) were multidrug resistant, out of 9 isolates of *A. johnsonii*, 8 (88.8%) were multidrug resistant and all 6 (100%) isolates of *A. lwoffii* were multidrug resistant (Table 2). One from each *A. baumannii* and *A. johnsonii* isolates was susceptible to co-amoxiclav; all 6 isolates of *A. lwoffii* were resistant to it. Overall, only 2% of isolates were susceptible to co-amoxiclav. Twenty eight (32.94%) isolates of *A. baumannii* and one (11.11%) of *A. johnsonii* were susceptible to ampicillin-sulbactam, all 6 (100%) isolates of *A. lwoffii* were resistant to it. Overall, 29% isolates were susceptible to ampicillin-sulbactam. Forty one (48.23%) isolates of *A. baumannii* and one (11.11%) isolate of *A. johnsonii*, were susceptible to piperacillin-sulbactam, all 6 (100%) isolates of *A. lwoffii* were resistant to it. Overall, 42% isolates were susceptible to piperacillin-sulbactam. Eight (9.41%) isolates of *A. baumannii* and one (11.11%) isolate of *A. johnsonii*, were susceptible to piperacillin-tazobactam, all 6 (100%) isolates of *A. lwoffii* were resistant to it (Figure 1). Overall, 9% isolates were susceptible to piperacillin-tazobactam. Forty eight (56.47%) isolates of *A. baumannii* and one (11.11%) isolate of *A. johnsonii*, were susceptible to cefoperazone-sulbactam, all 6 (100%) isolates of *A. lwoffii* were resistant to it. Overall, 49% isolates were susceptible to cefoperazone-sulbactam (Figure 2).

DISCUSSION

In this study, cefoperazone-sulbactam among β-lactam - β-lactamase inhibitors was the most effective combination against 49% of Acinetobacter isolates. Nighty eight percent of total isolates were resistant to co-amoxiclav, 71% isolates were resistant to ampicillin-sulbactam, 58% isolates were resistant to piperacillin-sulbactam and 91% isolates were resistant to piperacillin-tazobactam. Combinations having sulbactam were more effective as compared to others. These results also supports the postulate that sulbactam, though not antimicrobial but does possess antibacterial activity against Acinetobacter species (Visalli et al., 1997).

A local study in 2012 showed that antimicrobial resistance in *Acinetobacter ssp*. is on rise. 46 isolates of *Acinetobacter spp.* were included in that study. 30.4% isolates were susceptible to ceftriaxone, 67.4% isolates were susceptible to cefepime, 56.5% isolates were susceptible to ciprofloxacin, 82.6% isolates were susceptible to both imipenem and meropenem. 23.9% of isolates were susceptible to co-amoxiclav as compared to 2% isolates of our study, 78.0% of isolates were susceptible to piperacillin-tazobactam as compared to 9% isolates of our study, 93% of isolates were susceptible to cefoperazone-sulbactam as compared to 49% of our study.

In that study all isolates were from blood culture specimens while in our study all kinds of clinical specimens

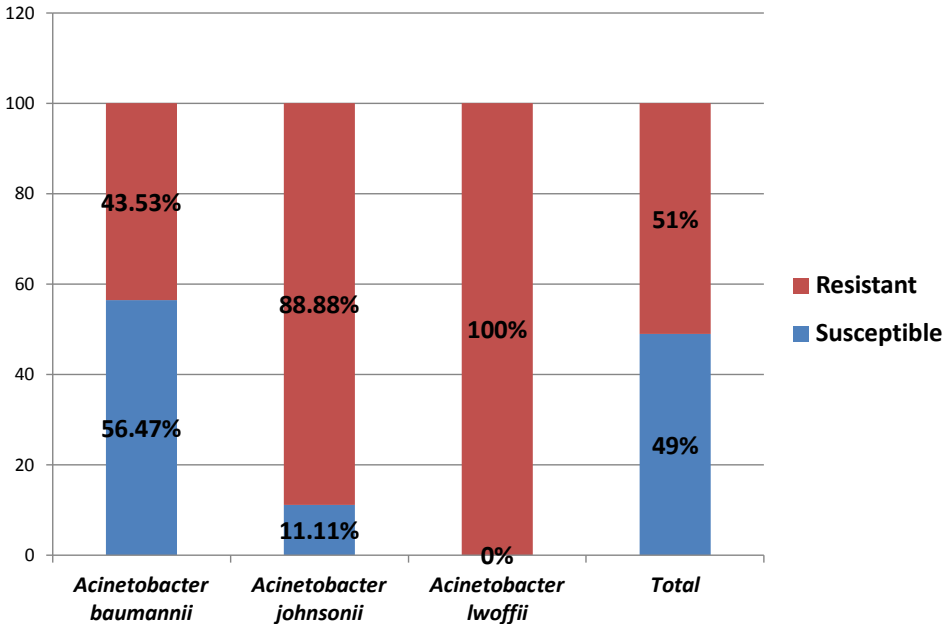

Figure 2. Susceptibility of Acinetobacter isolates to cefoperazone-sulbactam

were included (Javed et al., 2012).

In a study from USA in 1997, 3 combinations of β-lactam-β-lactamase inhibitors were tested against Acinetobacter species. In that study, 86.9% isolates of *Acinetobacter* spp. were susceptible to ampicillin-sulbactam, while in our study only 29% of isolates were susceptible to this combination. Their 84.8% isolates of *Acinetobacter* spp. were susceptible to piperacillin-tazobactam, while in our study only 9% of isolates were susceptible to it. Their 54.4% isolates were susceptible to co-amoxiclav, whereas only 2% of our isolates were susceptible to it. The other two combinations were not tested in that study. As compared to previous study our study has decreased susceptibility pattern; possible reason for that previous study is that it was conducted almost 16 years ago and *Acinetobacter* spp. has acquired resistance over time (Seward et al., 1998).

In a study from Germany in 2004, 115 isolates of *A. baumannii* were tested against different combinations of β-lactam - β-lactamase inhibitors. In that study, 35.6% isolates of *A. baumannii* were susceptible to co-amoxiclav as compared to 2% isolates of our study, 87.2% isolates of *A. baumannii* were susceptible to ampicillin-sulbactam as compared to 29% isolates of our study, 70.1% isolates of *A. baumannii* were susceptible to piperacillin-tazobactam as compared to 9% isolates of our study, 91.8% isolates of *A. baumannii* were susceptible to cefoperazone-sulbactam as compared to 49% of our study (Higgins et al., 2004). A common finding in our study with that of Higgins et al. (2004) that cefoperazone-sulbactam was the most effective drug against Acinetobacter, although time difference between 2 studies is almost 10 years. One difference between the two studies is that in German study 100% isolates were of *A. baumannii,* while in our study 85% isolates were of *A. baumannii* (Higgins et al., 2004).

In a study from Germany in 2005, 469 isolates of *Acinetobacter* spp. were tested against 6 different β-lactam - β-lactamase inhibitors combinations. In that study, 33.9% isolates of *A. baumannii* were susceptible to co-amoxiclav as compared to 2% isolates of our study, 90.9% isolates of *A. baumannii* were susceptible to ampicillin-sulbactam as compared to 29% isolates of our study, 79.7% isolates of *A. baumannii* were susceptible to piperacillin-tazobactam as compared to 9% isolates of our study, 91.4% isolates of *A. baumannii* were susceptible to piperacillin-sulbactam as compared to 42% of our study. Piperacillin-sulbactam was most effective combination susceptible to 91.4% of isolates as compared to our cefoperazone-sulbactam susceptible to 49% of isolates (Brauers et al., 2005).

In 2013, a study was conducted in Malaysia on 141 isolates of *Acinetobacter spp*. They tested different combinations of β-lactam - β-lactamase combinations but not all combinations which are included in our study. 14.2% isolates of *Acinetobacter* spp. were susceptible to co-amoxiclav as compared to 2% isolates of our study, in both studies 29% isolates of *Acinetobacter* spp. were susceptible to ampicillin-sulbactam, 23% isolates of *Acinetobacter* spp. were susceptible to piperacillin-tazobactam as compared to 9% isolates of our study, 29.1% isolates of *Acinetobacter* spp. were susceptible to cefoperazone-sulbactam as compared to 49% of our study. Results of both studies are comparable and it may

be because of the same time period (Biglari et al., 2013).

Although resistance is emerging against β-lactam - β-lactamase combinations in *Acinetobacter* spp. but combinations containing sulbactam are still more effective as compared to other combinations and may represent an effective therapeutic option.

Conflict of interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Identification of volatile compounds, antimicrobial properties and antioxidant activity from leaves, cones and stems of *Cupressus sempervirens* from Algeria

Nafila Zouaghi[1]*, Cherifa Bellel[1], Carlos Cavaleiro[2], Boubekeur Nadjemi[1] and Mohamed Yousfi[3]

[1]Laboratoire d'étude et de développement des techniques de traitement et d'épuration des eaux et de gestion environnementale. Ecole Normale Supérieure, Kouba, Algeria.
[2]Centro de Estudos Farmacêuticos / Faculdade de Farmácia, Universidade de Coimbra, Portugal.
[3]Laboratoire des Sciences Fondamentales, Université Amar Telidji, Laghouat, Algeria.

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Cupressus sempervirens L. **(Cupressaceae) leaves, cones and young branches have been used in traditional medicine and aromatherapy. The composition of the isolates obtained by hydrodistillation from the aerial parts (plant material collected at Bainam forest, in Northwest of Alger, Algiers), were analyzed by GC and GC-MS. The leaves isolate (yield 0.22% w/w), was mainly composed of monoterpene hydrocarbons (60.8%), α-pinene (38.4%), δ-3-Carene (13.9%), α-Cedrol (10.6%), α-Terpinyl acetate (3.5%) and E-Totarol (3.0%). The cones isolate (yield 0.34% w/w) was predominantly composed of monoterpene hydrocarbons (33.I8%), with α-pinene (20.3%), δ-3-Carene (6.0%), Tepinene-4-ol (9.0%), α-Terpineol (9.0%), α-Terpinyl acetate (5.9%), α-Cedrol (9.1%), and E-Totarol (4.4%). The major components of stems isolates (yield 0.03% w/w) are rich in diterpenoids (51.9%), namely: α-pinene (5.9%), α-Cedrol (14.4%), Manool (5.6%), E-Totarol (34.7%), Ferrugenol (6.0%). Isolates were also tested against four bacteria (*Bacillus subtilis, Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*), and two yeasts (*Saccharomyces cerevisiae* and *Candida albicans*), using the Kirby Bauer disk-diffusion method. All bacteria were susceptible to the *C. sempervirens* volatiles isolates. Antioxidant activity of the isolate was evaluated by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) method together with two antioxidant standards, butylated hydroxytoluene (BHA) and tert-butyl-4-hydroxy toluene (BHT). The results show antioxidant effect of all isolates less significant as BHA and BHT.**

Key words: *Cupressus sempervirens*, GC/SM, chemical composition, Antimicrobial activity, Antioxidant activity, using 1, 1-diphenyl-2-picrylhydrazyl (DPPH).

INTRODUCTION

The genus *Cupressus* (Cupressaceae) consists of 12 species spread across North America, the Mediterranean basin, and subtropical Asia at high altitudes. Three species were reported as part of North African flora, for convenience they were called *Cupressus sempervirens aggr;* are often confused, being closely related and similar in external appearance (Greuter et al., 1984). These aggregate species include Algerian endemic

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

species *C. dupreziana*, *A. Camus*, and *C. sempervirens L*. (Neffati et al., 1999). *C. sempervirens* is considered to be a medicinal tree, as its dried leaves are used as an emmenagogue and for stomach pain (Castro, 1998), as well as to treat diabetes, and its dried fruit is used to treat inflammation, toothache, and laryngitis as a contraceptive and astringent (Mascolo et al., 1987). In addition, its dried seeds have been used to treat wounds, ulcers, bruises, sores, pimples, pustules, skin eruptions, and erysipelas (Caceres et al., 1987), the branches of this plant are used as antiseptics and antispasmodics (Bellakhder, 1997). The essential oil from the leaves and cones is used externally for headache, colds, cough, and bronchitis. With respect to these medicinal and pharmacological advantages, *C. sempervirens* is widely used as a cosmetic ingredient in perfumery and soap-making, including its essential oil distilled from shoots (Usher, 1974). *C. sempervirens* is very widespread in Algeria and in the entire Mediterranean region. This species has many specific botanical features, including tolerance to drought, air currents, wind-driven dust, sleet, atmospheric gases, a well-developed root system, the ability to flourish in both acidic and alkaline soils (Imededdine, 2013).

Several studies on the chemical composition of the essential oil of *C. sempervirens* have been previously reported (Ulukanli et al., 2014). The chemical composition of the essential oil from resin and its Biological activity were studied (Rawat et al., 2010). A detailed study aimed to investigate the chemical composition of fruits (Herzi et al., 2013) have compared the chemical composition and antioxidant activity of essential oil of leaves obtained by hydrodistillation and supercritical extraction. However, almost all of the published studies have examined the chemical composition of the essential oil of the leaves, fruit and stems separately. Therefore, the aim of this work was to study the composition of essential oils from tree organs: leaves, stems and cones, of horizontalys *C. sempervirens* that grow in Algeria and the measure of the antimicrobial and antioxidant activities.

MATERIALS AND METHODS

Plant material and essential oil preparation

Plant material was collected at Bainem forest (May, 2011), in Northwest of Algiers, Algeria. All parts of the plant were dried at room temperature, in the dark, for 15 days. The material was used for hydrodistillation using a Clevenger type apparatus, during 5 h, following the European Pharmacopoeia procedure (Council of Europe, 1997). Condensed volatiles were then recovered from the hydrolyte by extraction with diethyl oxide. Solvent was further eliminated under a gentle stream of nitrogen, rendering extracts with intense odours respectively at yields of 0.22% (w/w); 0.03% (w/w); 0.34% (w/w) for leaves, stems and cones.

Analysis

The volatile isolated were analyzed by gas chromatography (GC) (Hewlett-Packard 6890) equipped with a single injector and two flame ionization detection (FID) systems. For simultaneous sampling, two Supelco fused silica capillary columns with different stationary phases: SPB-1 (polydimethylsiloxane 30 m × 0.20 mm i.d. film thickness 0.20 µm), and SupelcoWax-10 (polyethyleneglycol) were used. The oven temperature program was: 70-220°C (3°C.min-1), 220°C (15 min); injector temperature: 250°C; carrier gas: helium; splitting ratio 1:40; detectors temperature: 250°C. GC-MS was carried out in a Hewlett-Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane 30 m × 0.25 mm i.d., film thickness 0.25 µm). GC parameters were as described above; interface temperature: 250°C; MS source temperature: 230°C; MS quadruple temperature: 150°C; ionization energy: 70 eV; ionization current: 60 µA. Compounds were identified by their GC retention indices on both SPB-1 and SupelcoWax-10 columns and from their mass spectra. Retention indices, calculated by linear interpolation relative to retention times of C8-C23 of n-alkanes (Van den Dool and Kratz, 1963), were compared with those of reference samples included in C.E.F. / Faculty of Pharmacy, University of Coimbra laboratory database. Acquired mass spectra were compared with reference spectra from the laboratory database (Wiley, 2005) and validated literature data (Adams, 2004; Joulain and Koenig, 1998; Cavaleiro et al., 2011). Relative amounts of individual components were calculated based on GC raw data areas without FID response factor correction.

Antimicrobial activity

The essential oils obtained from the aerial parts of C*. sempervirens* were tested against four bacteria (reference strains): *Bacillus subtilis* ATCC 9372, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 4157, and two yeasts: *Saccharomyces cerevisiae* ATCC 601, *Candida albicans* ATCC 24433 strains. The microbial strains were supplied by SAIDAL (pharmaceutical group). The bacteria strains were inoculated into nutrient broth Muler-Hinton (30°C) and incubated for 24 h. Based on modern culture the bacterial suspension was obtained for 18-24 h (48 h for yeasts). 3-5 bacterial colonies were taken far away from each isolate and placed in 6 ml of sterile physiological water. The focus 10^6 CFU/ml for wavelength 450 nm was obtained (Hammer et al., 1999). The paper discs (6 mm in diameter) were separately impregnated with different concentration (20, 100 and 300 µg) of the oil dissolved in Dimethylsulfoxide DMSO (Sigma Aldrich) and placed on the nutrient broth, which had previously been inoculated with the selected test microorganism, respectively. Plates, after 1 h at 4°C, were incubated for bacteria at 37 ◦C for 24 h and for yeasts strains at 30°C for 48 h. The DMSO solvent was used as the negative control. Standard antibiotics (25 µg/disk) Sulfamethoxazol-trimethoprim, Cefixim, Amoxicillin and Lymecyclin were used as positive controls. Antimicrobial activity was assessed by measuring the diameter (DD) of the growth inhibition zone in millimetres (including disc diameter of 6 mm). Tests were carried out in triplicate.

Antioxidant activity

The antioxidant activity was measured using the DPPH assay (Brand-Williams et al., 1995). 1000 µg of essential oils and tested substances in ethanol were added to 3 ml of 0.004% ethanol solution of DPPH. After 30, 60, 90 min at 24 h incubation period at room temperature, the absorbance was read against a blank at 515 nm. Inhibition free radical DPPH in percent (I%) was calculated as: $I\% = \frac{(Ac-Ae)}{Ac} \times 100$; where, Ac was the absorbance of the control reaction (containing all reagents except the test compound), and Ae was the absorbance of the test compound.

Table 1. Volatiles components of the leaves (L), cones (C) and stems (S) of *Cupressus sempervirens*.

RI[a]	RI[b]	Compound	Percent in samples (%)		
			Leaves	Cones	Steams
I924	1030	α-Thujene	0.3	-	0.2
933	1034	α-Pinene	38.4	20.3	5.9
942	1067	α-Fenchene	1.0	0.3	t
966	1127	Sabinene	0.3	-	0.9
970	1118	β-Pinene	1.0	0.9	0.2
982	1163	Myrcene	1.3	1.3	0.5
1007	1156	δ-3-Carene	13.9	6.0	3.0
1011	1185	α-Terpinene	0.2	-	0.3
1013	1274	*P*-Cymene	0.1	-	0.3
1021	1206	Limonene	-	1.5	0.4
1021	1215	β-Phellandrene	2.0	-	t
1047	1248	γ-Terpinene	0.3	0.5	0.2
1048	1456	*Z*-Sabinene hydrate	-	-	0.1
1078	1287	Terpinolene	1.9	2.9	2.2
1084	1539	*E*-Sabinene hydrate	-	-	1.1
1084	1539	Linalool	0.2	-	t
1100	1595	Fenchyl alcool	-	0.1	-
1109	1555	*Z*-p-2-menthen-1-ol	-	0.4	-
1121	1620	*E*-p-2-menthen-1-ol	-	0.2	-
1130	1566	Pinocarvone	-	0.5	-
1145	n.d.	3-Thujene-2-one	0.1	-	-
1147	1664	Borneol	-	2.1	-
1159	1600	Tepinene-4-ol	0.8	9.0	2.1
1170	1692	α-Terpineol	1.0	9.0	t
1226	1598	Carvacrol methyl ether	-	-	0.6
1266	1574	Bornyl acetate (endo)(L)	0.1	1.5	0.3
1332	1692	α-Terpinyl-acetate	3.5	5.9	1.8
1343	1456	α-Cubebene	0.1	0.8	0.9
1368	1488	α-Copaene	-	0.2	-
1394	1567	Longifolene	-	0.1	0.4
1400	n.d.	Iso-allo-longifolene	-	1.9	-
1400	1565	α-Cedrene	0.3	-	-
1406	1591	β-Cedrene	0.2	-	-
1410	1595	Caryophyllene	-	3.4	-
1447	1664	α-Humulene	0.2	-	-
1461	1681	γ-Muurolene	0.2	-	t
1465	1703	Germacrene D	-	2.6	T
1488	1719	*Z*-α-Bisabolene	-	0.5	-
1496	1748	γ-Cadinene	0.2	0.4	-
1506	1748	δ-Cadinene	0.7	0.3	-
1518	1908	α-Calacorene	-	0.4	-
1557	1978	Caryophyllene oxide	0.1	1.2	-
1579	2108	α-Cedrol	10.6	9.1	14.4
1591	2143	Cedrol epi	0.7	-	T

RESULTS AND DISCUSSION

Chemical composition of the essential oils

The composition of the leaves, cones and stems distillates are summarized in Table 1. The detected compounds are listed according to their elution from GC (SPB-1 column) (Figures 1 to 3). The leaves distillation rendered a pale yellow liquid 0.22% (w/w) four times

Table 1. Contd.

RI[a]	RI[b]	Compound	Percent in samples (%)		
			Leaves	Cones	Steams
1607	2154	γ-Eudesmol	-	0.3	-
1615	2188	α-Muurolol	0.7	-	-
1622	2212	β-Eudesmol	-	0.4	-
1627	2220	α-Cadinol	1.6	-	-
1655	2161	α-Bisabolone oxide	1.6	-	-
1964	2343	Manyol oxide	0.3	-	1.1
1968	2299	Isopimaradiene	0.6	0.2	-
1987	n.d.	Manoyl oxyde 13-epi	-	-	0.4
1988	2331	Cyperone alpha	-	0.2	-
2017	2469	Abietatriene	0.5	-	2.2
2026	n.d.	Manool	-	0.6	5.6
2069	n.d.	Isoabienol	0.7	0.3	-
2232	n.d.	*Z*-Totarol	-	0.8	1.8
2255	n.d.	*E*-Totarol	3.0	4.4	34.7
2273	n.d.	Ferruginol	0.5	0.7	6.0
Monoterpene hydrocarbons			60.8	33.8	13.0
Oxygen containing monoterpenes			2.1	21.4	4.9
Sesquiterpene hydrocarbons			5.8	17.9	3.3
Oxygen containing sesquiterpene			15.3	11.0	14.4
Diterpenoids			5.6	7.3	51.9
Total identified			89.6	91.4	87.5

Compounds listed in order their elution from SPB-1 column: RIa, Retention indice in the SPB-1 column; Rib, Retention indice in the Supelcowax 10 column; n.d, non-determined; t, traces (<0.05%).

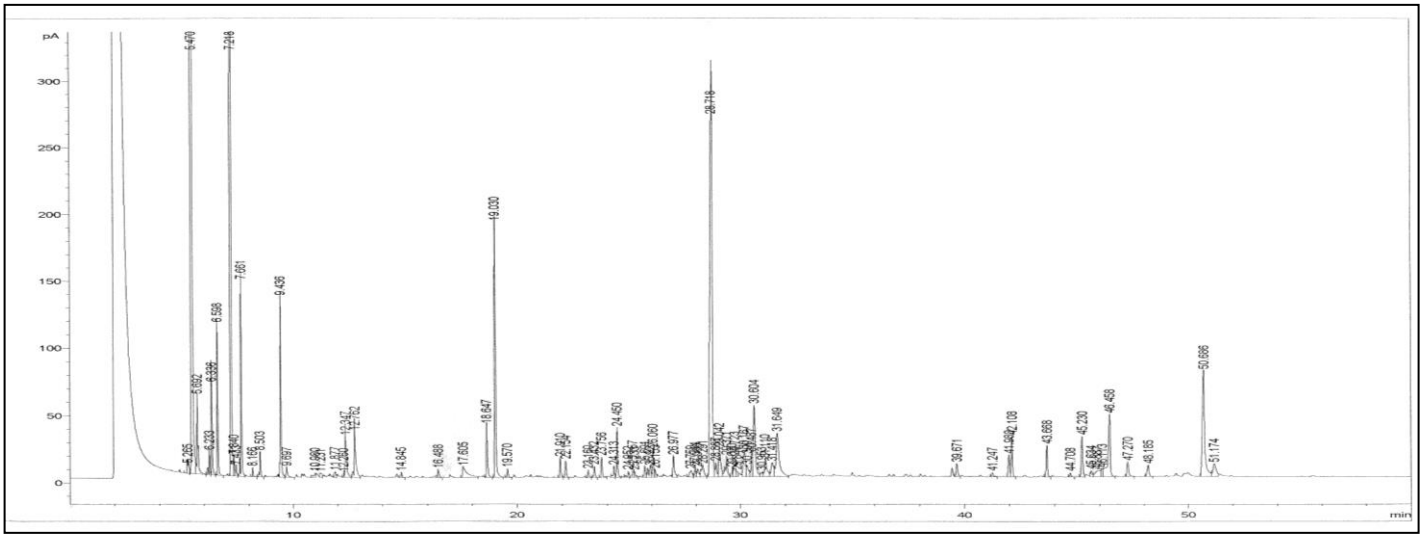

Figure 1. Chromatograms and main compounds of the essential oil of leaves from *C. sempervirens* obtained by SPB-1 column.

inferior than the Cameroonian 1% (w/w) (Tapondjou et al., 2005). In this isolate, 37 components (89.6%) were identified of which α-Pinene, (38.4%) followed by δ-3 Carene (13.9%), α-Cedrol (10.6%), α-Terpinyl acetate (3.5%), E-Tatarol (3.0%) and β-Phellandrene (2.0%), were the most abundant. Monoterpene hydrocarbons contribute to (60.8%), for the global composition. The major constituents were com-pletely different from those of previous report on the chemistry of these oils (Hosni et al., 2014).

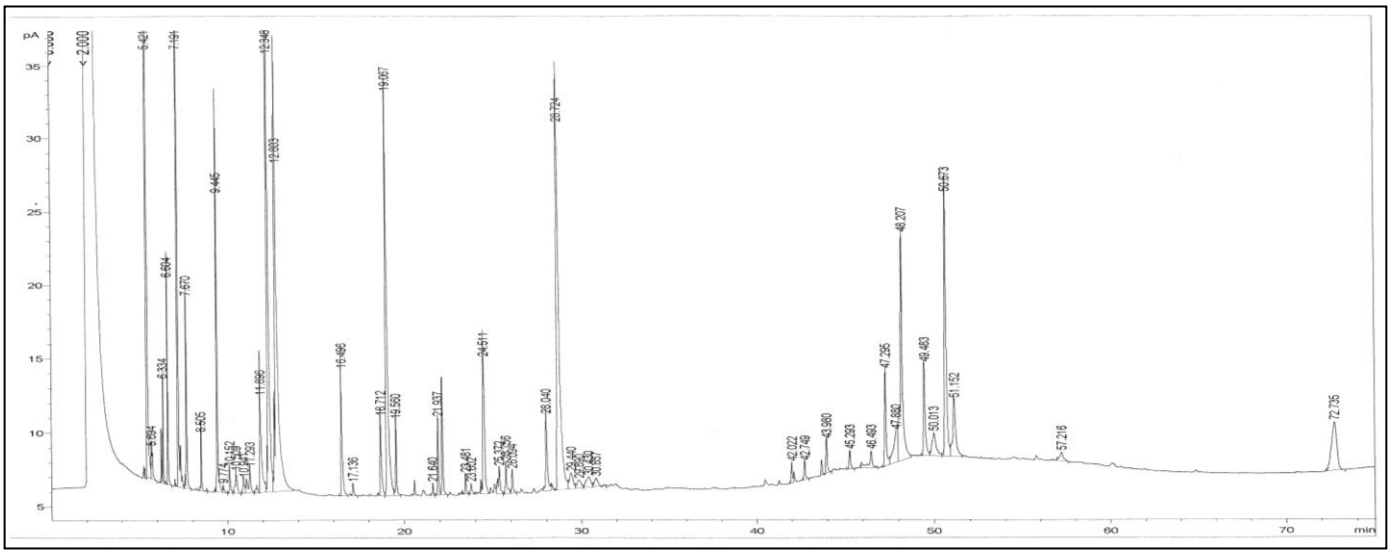

Figure 2. Chromatograms and main compounds of the essential oil of cones from *C. sempervirens* obtained by SPB-1 column.

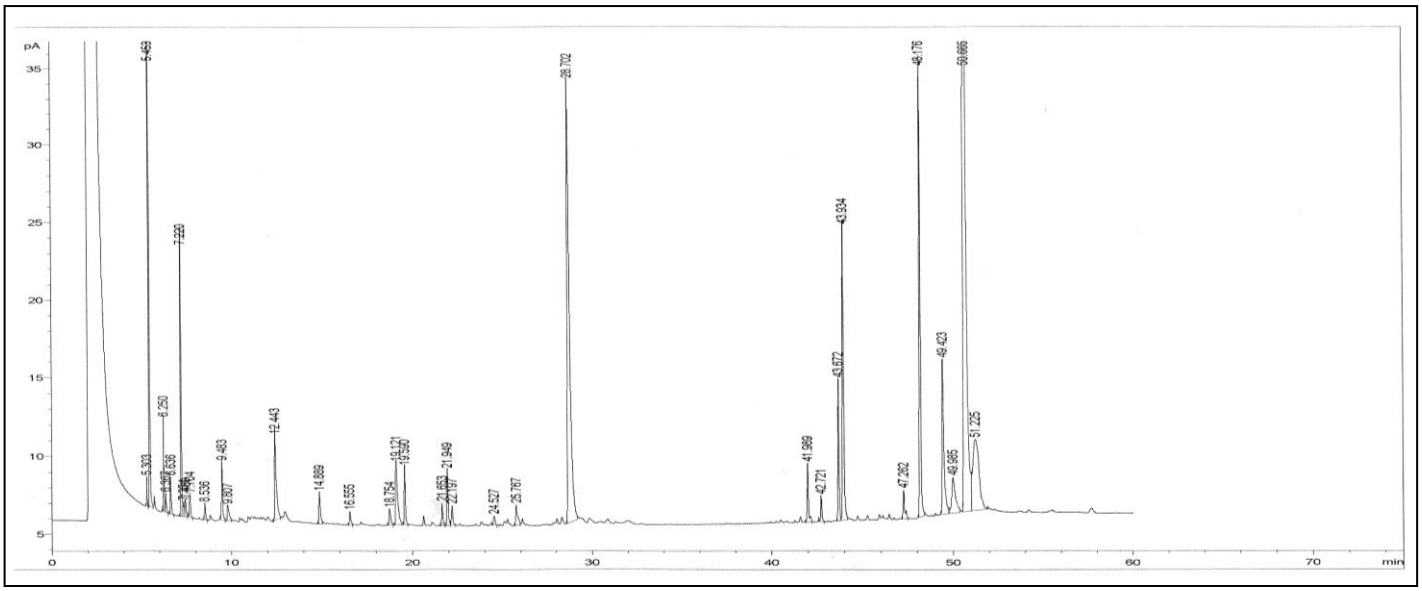

Figure 3. Chromatograms and main compounds of the essential oil of stems from *C. cempervirens* obtained SPB-1 column.

The chemical composition of isolated essential oils from leaves are in accordance with those previously reported (Amri et al., 2013). Thirty-eight (38) components were identified (91.4%) in the cones isolate. The most abundant constituents were the monoterpenes hydrocarbons (33.8%): α-Pinene (20.3%) and δ-3 Carene (6.0%), the monoterpenes oxygenes (21.4%): α-Terpinyl acetate (5.9%) and the oxygenateds sesquitepenes (11%): α-Cadinol (9.1%). The stems isolate was chiefly composed of deterpinoids (51.9%) with E-Tatarol (34.7%) and α-Cedrol (14.4%) as major components. The amount of α-Pinene and δ-3 Carene were lower in cones and stems than leaves isolates, decreasing from 60.8 to 33.8, 13.0 and from 13.9 to 6.0, 3.0%, respectively.

Antioxidant activity

The results obtained during the test measurement of the percentage of inhibition of DPPH radical are shown in Figure 4. It seems that the percentage inhibition of free radicals increased with increasing time for the essential oil of *C. sempervirens*. The inhibition percentage of free radicals for the essential oil is lower than that of BHA and BHT. These results show that the essential oil of *C.*

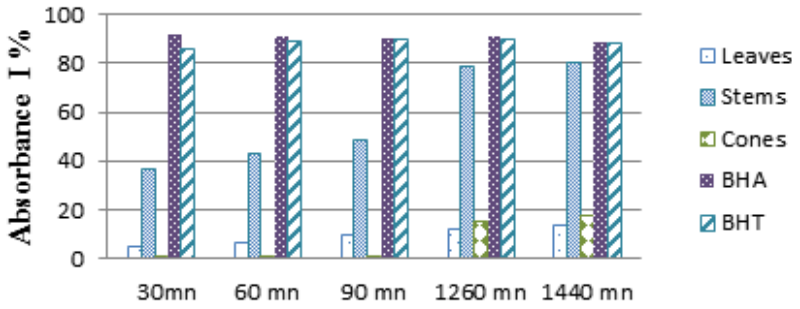

Figure 4. The reducing power of various times of: BHA, BHT, leaves, cones, stems.

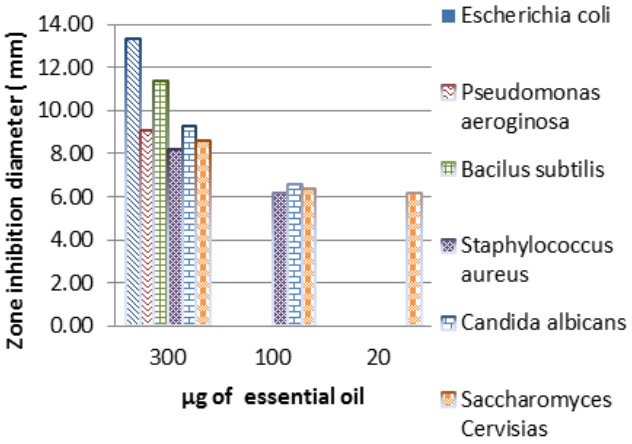

Figure 5. Antimicrobial activities of the essential oil of leaves.

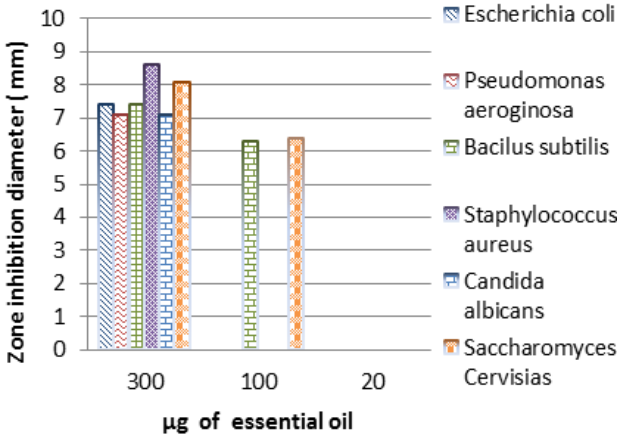

Figure 6. Antimicrobial activities of the essential oil of cones

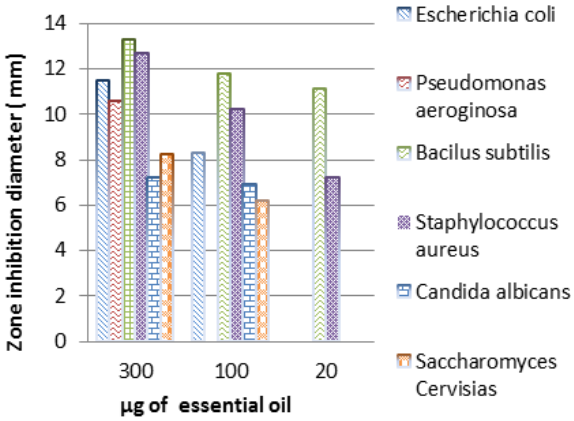

Figure 7. Antimicrobial activities of the essential oil of stems

sempervirens has antioxidant activity in concentration of 1000 µg but it is less effective than BHA and BHT for leaves and cones. As shown in Figure 1, the inhibition activity of the stems essential oils was same to the activity of BHA and BHT. This may be due to the presence of diterpinoids (51.9%) specifically to E- Tatarol which is a major component of the essential oil studied (34.7%) and has a strong antioxidant activity (Haraguchi et al., 1997). Moreover, the presence of carvacrol even at low concentrations in the stems essential oil (0.6%) may explain the scavenging activity of DPPH radical.

Antimicrobial activity

The antibacterial activity of the essential oils was evaluated against four microorganisms and two yeast, using disc diffusion methods. The disc diameters (DD) of essential oils inhibition zone for the tested microorganisms are shown in Figures 5 to 7. Results show that all the oils inhibited the growth of microbial strains. A zone diameter of inhibition from 6.2 to 13.3 mm was observed, depended on susceptibility of the tested microbial. The oil of leaves, cones and stems have a high antimicrobial activity against the bacterial strains and yeasts at a concentration of 300 µg, however, at this concentration the oil extracted from leaves did not inhibit *P. aeruginosa*. The leaves showed better inhibitory effect on *E. coli* and *S. Cerevisiae* when compared to the cones and stems. The presence of α-Pinene (38.4%) and δ-3 Carene (13.9%) in leaves isolate are attributed the antimicrobial (Guy et al., 2001; Jiang et al., 2011; Ojeda-

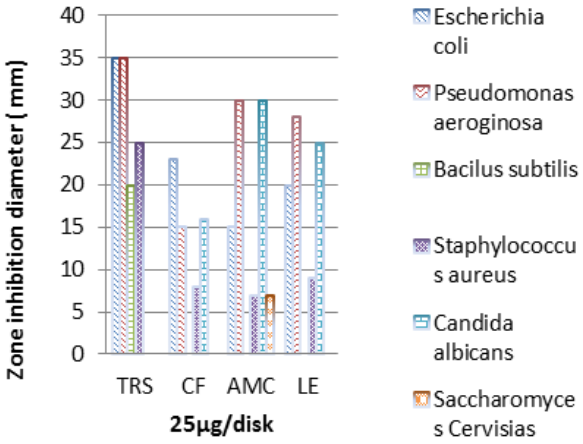

Figure 8. Antimicrobial activities of standards antibiotics. TRS, Trimethoprim-sulfamethoxazol; CF, cefixim; AMC, Amoxicillin; LE, Lymecyclin.

Sana et al., 2013; Hmamouchi et al., 2001). The presence of Tepinene-4-ol in the stems and cones with respectively 2.1 and 9.0% give excellent inhibition against S*. aureus* and *P. aeruginosa* (Jirovetz et al., 2005).

Stems have a good inhibitory activity against *B. subtilis* compared to cones and leaves; diterpinoids (51%) are effective against bacteria (Kotan et al., 2007). The antimicrobial activity of the essential oils extracted from different parts of C*. sempervirens* are, in part, associated with their major constituents such as α- Pinene, β-Phellandrene, α-Terpinyl acetate and Cedrol. These components have been reported to display antimicrobial effects (Cosentino et al., 1999; Alessandra et al., 2005; Yang et al., 2007; Demirci et al., 2007).

The essential oils containing terpenes are also reported to possess antimicrobial activity (Dorman and Deans, 2000), which are consistent with our present study. *Saccharomyces cerevisiae* exhibited high resistance to any standard antibiotic; however its activity was inhibited by the essential oils extracted from different parts of the plant (Figure 8).

Conclusion

Chromatographic analyses have identified 37 and 38 components with a codominance of α-pinene (38.4 and 20.3%) for leaves and cones, respectively, and 34 components with E-Tatarol (34.7%) as the major component for the stems. The results obtained in this work show that the C*. sempervirens* essential oils possess antimicrobial properties, which can be used as natural antimicrobial agents for human and infectious diseases and in food preservation. The stems essential oil exhibited a better free radicals inhibition compared with leaves and cones and was same microbiological activity that BHA and BHT.

Conflict of interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Obtaining DNA from *Staphylococcus aureus*: A study on DNA extraction methods for food matrices without bacterial isolation

Marjory Xavier Rodrigues[1]*, Gabriela Sartori Felkl[2], Renata Louize Samulak[2], Andiara Gonçalves Tenório[3] and Juliana Vitória Messias Bittencourt[2]

[1]Agroindustry, Food and Nutrition Department, Agriculture School "Luiz de Queiroz", University of São Paulo, +55 (19) 3429-4100, 11, Pádua Dias Ave., Piracicaba, Brazil.
[2]University Federal Technological of Paraná, Monteiro Lobato Ave., km 04, Ponta Grossa, Brazil.
[3]University Federal Technological of Paraná, Boa Esperança, km 04, Dois Vizinhos, Brazil.

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Polymerase chain reaction (PCR) is a tool widely used. However, for the effective use of PCR, DNA quality is necessary. Thereby, the objective of this study was to evaluate DNA extraction methods of *Staphylococcus aureus*, for direct application in food. Five methods were tested using reference and food strains. The methods show different characteristics with steps of thermal lysis, enzymatic lysis, detergents and organic solvent applications. The amplification was carried out using the primers COAG2 and COAG3 and the visualizations of the DNA extracted and amplification products were performed by gel electrophoresis. The method with SDS was not satisfactory using reference strain. The methods based on the use of cetyl trimethyl ammonium bromide (CTAB), sodium chloride-Tris-EDTA (STE) and enzymes showed positive results. Methods 2 (thermal lysis) and 3 (with CTAB) were used for DNA extraction from food samples, without bacterial isolation, and the PCR was subsequently performed. These methods were easier to implement and they show low costs. Thus, the methods 2 and 4 allowed the amplification of the DNA extracted from *S. aureus* from samples rich in protein and fat. Method 2 is practical and shows other advantages such as less manipulation of samples and reagents, nonuse of contaminant reagents and enzymes, less time for analysis, thus, lower costs.

Key words: Thermal lysis, DNA, quality, polymerase chain reaction.

INTRODUCTION

Staphylococcal food poisoning is considered one of the most common foodborne illnesses (Pelczar et al., 1996). The number of cases has reduced, but it is the third cause of food borne illness worldwide (Aydin et al., 2011).Thereby, the importance of *Staphylococcus aureus* in medicine has increased and supported by the development of bacterial resistance antimicrobials and the capacity to produce enterotoxins (Vasconcelos and Cunha, 2010).

*Corresponding author. E-mail: marjoryxavier@usp.br.

This pathogen is detected, in most cases, using microbiological culture and biochemical tests; however, these methods are not entirely satisfactory (Cremonesi et al., 2005) because they are associated with ambiguous results (Pinto et al., 2005). In the last decades, molecular methods have been developed and recognized for detection and characterization of microorganisms in foods. Molecular biology is a viable alternative to conventional methods for pathogen diagnostics in foods.

The PCR stands out due to its specificity and sensibility, speed, easy handling, and is a useful tool for routine application (Pinto et al., 2005). To obtain success in the reaction, DNA quality is necessary. The quality and yield of extracted DNA are critical for most applications of molecular biology (Minas et al., 2001). There are many DNA extraction kits, but they are expensive for small laboratories. Therefore, it is necessary to develop or adapt extraction methods.

Most extraction methods are based on sample and microorganism characteristics and promote cell rupture, cell components separation and DNA precipitation (Lima, 2008).

The microorganism isolation is common; however, bacterial isolation prior to extraction increases time and cost of analysis. Thus, the direct DNA extraction is proposed. The objective of this study was to evaluate methods of DNA extraction from *S. aureus* for direct application in food. The use of bacterial DNA extracted directly from food sample, without bacteria isolation, is the main point in this search.

MATERIALS AND METHODS

Samples

Firstly, strains of *S. aureus* ATCC 6538P and ATCC 25923 were used and *S. aureus* isolated from milk denominated SA1, SA2 and SA3, respectively.

The food samples were fresh sausage, Calabresa sausage, Toscana sausage, Blumenau sausage and fresh thin sausage marketed in the city of Campos Gerais, Paraná State, Brazil. The samples were not artificially contaminated.

Before the DNA extraction the strains were grown in brain heart infusion (BHI) broth for 24 h at 37°C. The samples were inoculated in buffered peptone water for 24 h at 37°C for a better growth of *S. aureus* and for the inhibition of competing microorganisms, 8.5 g of NaCl per liter of water was added. This material was used for the DNA extraction after the incubation time without the isolation of *Staphylococcus* sp.

DNA extraction

Five different methods were evaluated as below:

Method 1: using SDS (sodium dodecyl sulfate 1%), proteinase K, chloroform: isoamyl alcohol and ethanol (Moreira et al., 2010) modified;
Method 2: based on the boiling (thermal lysis) without the use of specific reagents, cited by Chapman et al. (2001);
Method 3: described by Chapaval et al. (2006), using CTAB (cetyltrimethylammonium bromide 2%), proteinase K, chloroform: isoamyl alcohol, ethanol and isopropanol;
Method 4: based on Millar et al. (2000), use of chloroform/isoamyl alcohol and DNA precipitation with ethanol;
Method 5: using lysozyme, proteinase K, STE (2.5% SDS, 10 mM Tris-HCl, 0.25 M EDTA), ammonium acetate, chloroform: isoamyl alcohol and isopropanol (Luz, 2008).

All methods were evaluated using the strains SA1, SA2 and SA3. The methods that showed potential for direct extraction were applied in samples food. The DNA extracted from samples food were used for amplification to evaluate the quality of the extracted material and the possible influence of the compounds food.

DNA amplification

The primers used were COAG2 (5'ACCACAAGGTACTGAATCAACG3') and COAG3 (5'TGCTTTCGATTGTTCGATGC3') described by Aarestrup et al. (1995) cited by Luz (2008). The size of the amplified product is approximately 800 pb, because of polymorphism in this species. The PCR for the gene coa detection was conducted in a solution containing 1x PCR buffer, 0.75 mM $MgCl_2$, 1 µM of each primer, 200 µM of dNTPs, 1.5 U of Taq polymerase and approximately 40 ng of DNA; final volume was completed to 25 µL with sterile water.

The amplification conditions were: initial denaturation at 95° C for 5 min followed by 40 cycles of 95°C for 30 s, 55°C for 2 min and 72°C for 4 min, and final extension at 72°C for 10 min (Luz, 2008). The amplification was carried out in a thermal cycler Axygen Maxigene®.

Visualization DNA and PCR products

The DNA quality was visualized by electrophoresis in 0.8% agarose gel. The amplicons were visualized by standard electrophoresis in 1.5% agarose gel, with molecular size marker (100 pb). The gels were immersed in ethidium bromide (0.5 µg/mL) for 15 min. For the band visualization, gel exposure was performed in a UV transilluminator and image capturing by software LPix Image.

RESULTS AND DISCUSSION

The Gram's method is an important parameter for DNA extraction, because it indicates the cell wall composition and, hence, its resistance (Nogueira et al., 2004).

Baratto and Megiolaro (2012) tested four DNA extraction methods for positive Gram bacteria and observed that the use of the SDS method allied to proteinase K obtained high quality and greater quantity of DNA when compared with methods using only SDS or boiling methods. In this study, method 1 (with SDS and proteinase K) was not effective for all strains (Figure 1a). This result may be associated with the characteristics and origin of the strains. Furthermore, previous studies showed SDS for DNA extraction from Gram-negative bacteria (Gonçalves, 2006). On the other hand, to confirm that DNA was not extracted is not correct because the quantity can be so little that it is not visualized on agarose gel.

The presence of defined bands is a reliable parameter to establish the method efficiency. However, Nogueira et al. (2004) determined that the absence of bands defined in agarose gels is not a predictive factor for the PCR success. The method based on microorganism boiling in water and centrifugation was indicated for several bacteria.

Method 2 is faster, simpler and cheaper than others under

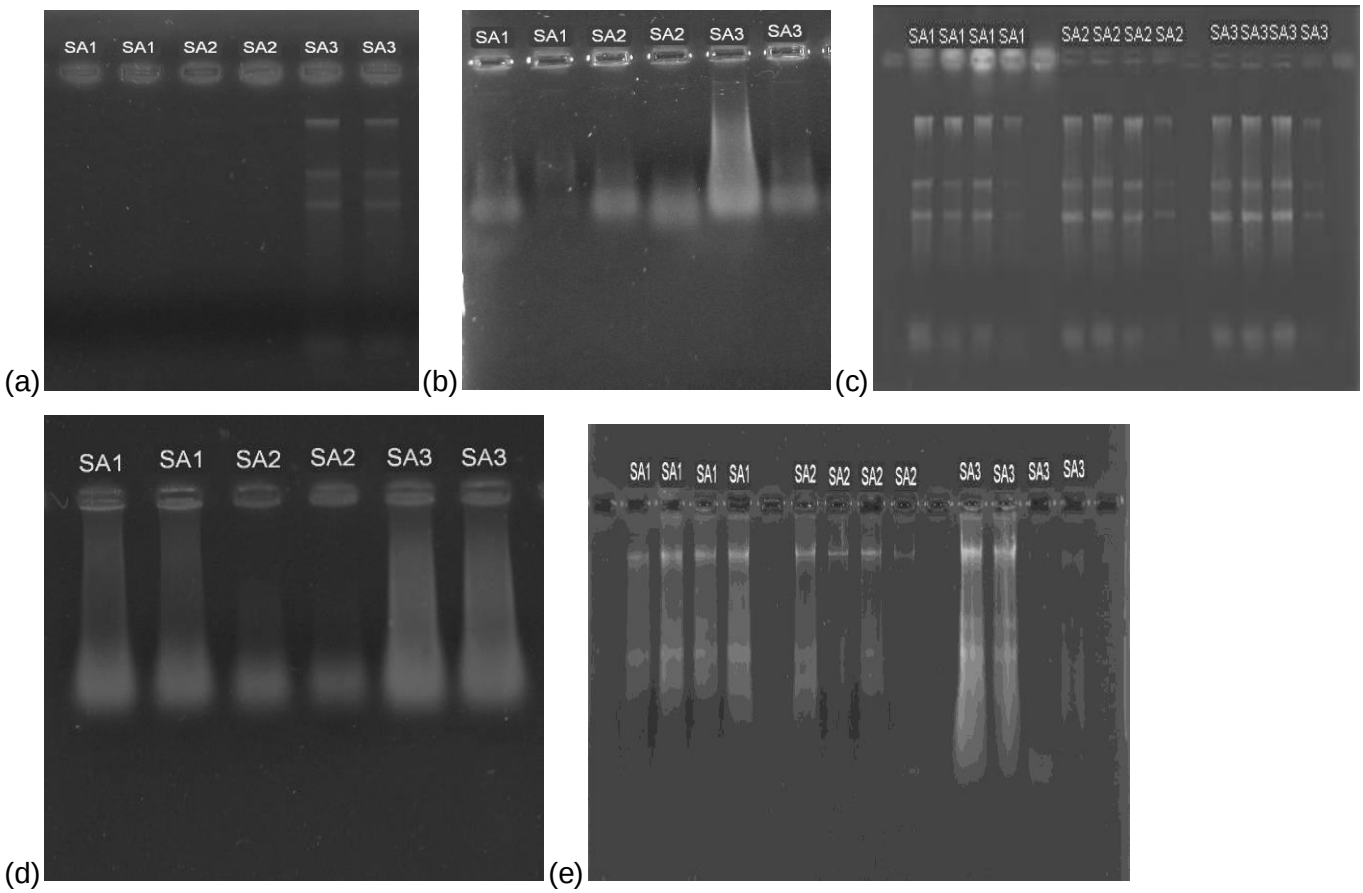

Figure 1. SA1: ATCC 6538P, SA2: ATCC 25923 and SA3: *S. aureus* isolated from milk. **(a)** Agarose gel with DNA extracted by Method 1. (b) Agarose gel with DNA extrated by Method 2. **(c)** Agarose gel with DNA extrated by Method 3. **(d)** Agarose gel with DNA extrated by Method 4; (e) Agarose gel with DNA extrated by Method 5.

discussion. But, the defined bands were not visualized (Figure 1b), because the DNA is only exposed and substances undesirables are present. Described by Chapaval et al. (2006) Method 3 was satisfactory (Figure 1c) for all strains, the CTAB is already widely used for DNA extraction from Gram positive bacteria Chapaval et al., 2006; Olivindo et al., 2009; Gonçalves et al., 2010; Minas et al., 2001).

The DNA obtained by Method 4 also showed no defined bands (Figure 1d); it was already used for detection of *S. aureus* directly from milk (Dias et al., 2011). Method 5 is ideal for *Staphylococcus* sp. (Figure 1e), but applies the use of various reagents and two enzymes, which increases the cost of the analysis.

Based on characteristics found, Methods 2 and 4 were selected for direct DNA extraction and subsequent PCR development due to low cost, although they showed no defined bands. If these methods do not present good results a next method would be tested. Thereby, after 24 h of incubation, the samples were subjected to DNA extraction and PCR.

The PCR results showed that the methods tested were able to extract DNA with adequate quality (Figure 2) and quantity; results were positive (coagulotypes with ~ 1000 bp). The result is noteworthy since the *S. aureus* DNA was extracted directly from food samples without cell isolation; these samples foods present high contents of protein, fat and seasonings, compounds that negatively affect in the PCR analysis.

Method 2 was considered the most suitable (Figure 2) because of the results and its advantages. This method presents advantages such as time of analysis, absence of the use of contaminant reagents and enzymes, less handling of samples and reagents and lower costs. Thus, Method 2 is indicated for direct extraction of food similar to those tested. However, the DNA extracted by this method is not suitable for storage, because rapid degradation occurs.

Conclusion

Methods employing CTAB, STE and enzymes showed positive results for DNA extraction from strains. Methods 2 and 4 were also tested in food products (sausages) and the DNA obtained were subjected to PCR, showing satisfactory results. In these cases, the DNA extraction of

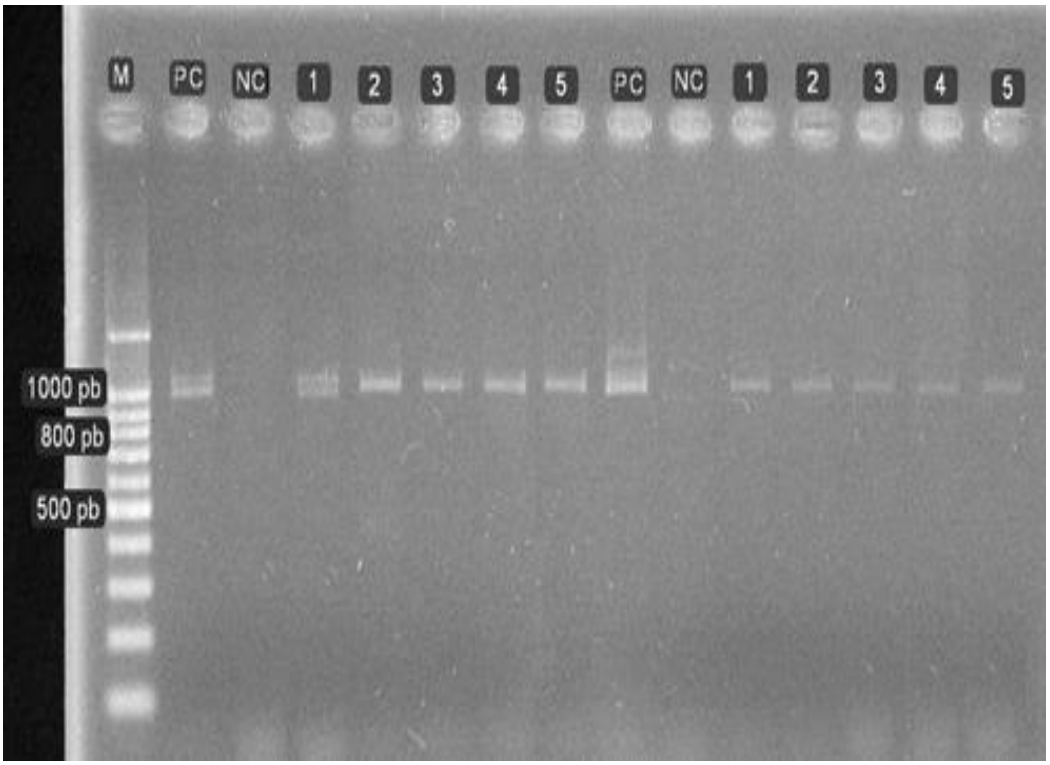

Figure 2. *coa* gene amplification from *S. aureus*. Line 2 to 8, DNA extraction by Method 2; Lines 9 to 15, DNA extraction by Method 4. M, molecular size marker; PC, Positive Control (ATCC 25923); NC, Negative Control (pure water); 1, fresh sausage; 2, Blumenau sausage; 3, Calabresa sausage; 4, fresh thin sausage; 5, Toscana sausage.

S. aureus directly from food in quantity and quality deserve highlight.

Diversified methods are available and the best choice should be linked to the quantity and quality of DNA extracted, analysis time, exposure to toxic reagents and costs. Thus, in this study, it can be concluded that Method 2 was the most suitable.

Conflict of interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Induction of defense-related proteins and growth promotion in tomato by mixture of *Trichoderma harzianum* OTPB3 and *Bacillus subtilis* OTPB1 and *Pseudomonas putida* OPf1 against *Phytophthora infestans*

S. P. Mohan Kumar[1], P. Chowdappa[1]*, V. Krishna[2] and H. Sandhya[1]

[1]Division of Plant Pathology, Indian Institute of Horticultural Research, Hessaraghatta Lake Post, Bangalore-560 089, India.
[2]Department of Post-Graduate Studies and Research in Biotechnology, Kuvempu University, Jnanasahyadri, Shankaraghatta 577 451 India.

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Late blight incited by *Phytophthora infestans* is a destructive disease of tomato worldwide. The plant growth-promoting antagonists, which elicit induced systemic resistance (ISR) and enhance plant growth, are being used as safe alternatives to synthetic fungicides for the management of plant diseases. In this study, a combination of *Trichoderma harzianum* OTPB3 and *Bacillus subtilis* OTPB1 and *Pseudomonas putida* OPf1 alone were evaluated for induction of systemic resistance in tomato against *P. infestans* in comparison with fungicides and growth promotion. Seed treatment with fresh suspensions of a combination of *T. harzianum* OTPB3 and *B. subtilis* OTPB1 caused significant increase in growth parameters compared to *P. putida* OPf1, mancozeb and untreated control due to higher production of indole-3-acetic acid (IAA) and gibberellic acid (GA_3). Reduction in the incidence of late blight was positively linked to increase of phenylalanine ammonia lyase, peroxidase, polyphenol oxidase and β-1,3-glucanase, the defense-related enzymes in tomato seedlings treated with microbial consortium of OTPB3 and OTPB1 followed by foliar spray of *P. putida* OPf1. The effects were on par with fenamidone and mancozeb treatments. The results reveal that seed treatment with microbial consortium containing *T. harzianum* OTPB3 and *B. subtilis* OTPB1 and foliar spray of *P. putida* OPf1 have practical significance in the management of late blight disease and also plant growth enhancement in tomato.

Key words: *Trichoderma harzianum, Bacillus subtilis, Pseudomonas putida, Phytophthora infestans*, Growth promotion, late blight, Growth hormones, induction of systemic resistance.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.), an important protective vegetable crop, is grown in 865,000 hectares in India (http://nhb.gov.in/area-pro/database-2011.pdf). Late blight, incited by *Phytophthora infestans* (Mont.) de Bary, is a devastating disease of tomato (*L. esculentum* Mill.), which often cause crop losses up to 100% (Fry et

al., 1993; Nowicki et al., 2012). *P. infestans* also causes root, foot, stem and fruit rot in addition to foliar blight in tomato (Lievens et al., 2004). The most commonly practiced methods to manage late blight are cultural, fungicide sprays, and use of resistant cultivars (Nowicki et al., 2012). Management of late blight using cultural practices alone is highly challenging particularly in areas, where tomato is grown year–round. Since commercial cultivars do not have adequate tolerance to late blight, chemical control involving fungicides scheduled at 5–7 days interval, form the basis for late blight management programs (Fry et al., 1993; Tumwine et al., 2002). Although fungicides have been successfully employed in managing late blight, their residues and environmental hazards leading to human health risks are major concerns. Development of resistance to fungicides by *P. infestans* further limits their use for disease management (Chowdappa et al., 2013a).

In recent years, biological control gained importance as an alternative to chemicals for plant disease management (Murphy et al., 2003; Woo et al., 2006; Harman, 2011). Biocontrol agents control the pathogens by several mechanisms which include direct antagonism, antibiosis, mycoparasitsm and siderophore production (Compant et al., 2005; Fridlender et al., 1993; Parke et al., 1991; Daayf et al., 1997). Besides, induced systemic resistance (ISR) in plants has been demonstrated as one of the modes by which biocontrol agents limit the effects of fungal infections (Schneider and Ullrich, 1994; Ramamoorthy et al., 2002; Saravanakumar et al., 2007; Latha et al., 2009; Chitrashree et al., 2011). Microbial consortia for plant growth enhancement and induction of systemic resistance (Janisiewicz, 1988; Choure et al., 2012) were successfully used. Janisiewicz (1988) reported antagonistic mixtures that exhibited biocontrol of post-harvest diseases in apple. Combination of three strains viz. *Pseudomonas fluorescens* LPK2, *Sinorhizobium fredii* KCC5 and *Azotobacter chroococcum* AZK2, suppressed the wilt incidence in *Cajanus cajan* (Choure et al., 2012) and enhanced plant growth due to synergism. Bio-consortium containing effective *Bacillus bassiana* and *P. fluorescens* strains controlled collar rot disease in groundnut both under polyhouse and field (Senthilraja et al., 2010).

Induction of defense responses by *Bacillus* spp., *Pseudomonas* spp. and *Trichoderma* spp. is largely related to increase of β-1,3-glucanase, phenylalanine ammonia-lyase, peroxidase, polyphenol oxidase and superoxide dismutase (Yedidia et al., 1999; Ahmed et al., 2000; Compant et al., 2005; Elad, 2000; Yang et al., 2009; Babitha et al., 2002). ISR incited by PGPR has been reported in many plants like *Arabidopsis* spp., bean, carnation, cucumber, radish, tobacco, and tomato (Van Loon et al., 1998). These biocontrol organisms control the diseases besides plant growth promotion through production of growth hormones like IAA and GA_3 (Chowdappa et al., 2013b). Systemic acquired resistance (SAR) against late blight was reported earlier in tomato by inoculating either pathogen (Christ and Mosinger 1989; Enkerli et al., 1993; Heller and Gessler, 1986) or by applying chemicals (Cohen, 1994) proceeding to confront the pathogen. ISR induced by PGPR has also been demonstrated in tomato against late blight incited by *Phytophthora infestans* (Yan et al., 2002). In our previous study, *Trichoderma harzianum* (OTPB3) and *Bacillus subtilis* (OTPB1) strains were identified that have the ability to induce systemic resistance against *Alternaria solani* and *P. infestans* (Chowdappa et al., 2013b) and also enhance plant growth. The aim of the present investigation was to know the additive effect of *T. harzianum* (OTPB3) and *B. subtilis* (OTPB1) strains as consortium and *Pseudomonas putida* (OPf1) individually through seed treatment in comparison to mancozeb followed by foliar spray of *P. putida* (OPf1) and fenamidone + macozeb for induction of systemic resistance in tomato against *P. infestans* and also plant growth promotion.

MATERIALS AND METHODS

Isolation and identification of biocontrol strains

Biocontrol strains *B. subtilis* OTPB1 and *T. harzianum* OTPB3 identified in our previous study (Chowdappa et al., 2013b) were used in this study. *P. putida* OPf1 was isolated from the rhizosphere soil sample from tomato crop at Ranga Samudrum, Andhra Pradesh, India using King's B Medium (King et al., 1954). Soil samples from rhizosphere were collected from healthy tomato plants grown under field conditions by uprooting plants carefully without any injury to the root system. Four plants from four different places were collected and the samples were mixed together and placed in polythene bags. Ten grams of soil was added to 90 ml of sterile distilled water and vigorously shaken for 10 min. The suspensions were serially diluted up to 10^{-7}. Then, 0.1 ml of 10^{-1}, 10^{-3}, 10^{-5} and 10^{-7} diluted samples was spread on King's medium B (King et al., 1954). Three replicate plates were incubated at 27±1°C for 48 h. After 48 h of incubation, all the isolates were checked for fluorescence under UV light at 365 nm (Sharifi-Tehrani et al., 1998). Colonies that showed fluorescence were selected and further purified on King's medium B agar medium. Pure isolates were stored at -80°C after addition of 30% glycerol (v/v).

DNA was isolated from 36 h old cultures of *P. putida* OPf1, grown in nutrient broth at 26±1°C, using bacterial DNA isolation kit (Zymo Research Bacterial DNA Mini Prep., USA). PCR amplification of 16S rDNA was performed using 27F (5'-AGAGTTTGATCCTGGCTCAG-3') (Weisberg et al., 1991) and 1492R-5´- GGTTACCTTACGACTT-3´ (Reysenbach et al., 1992). PCR was carried out in 50 µl reaction volumes. Each reaction consisted approximately of 1 µl of template DNA, 5 µl 10 x PCR buffer, 40.75 µl sterile distilled water, 1 µl 2.0 mM dNTPs, 1 µl each

*Corresponding author. E-mail: pallem22@gmail.com; pallem@iihr.ernet.in. Tel: +91-80-28466421. Fax: +91-80-28466291.

of 50 pM primers 27F and 1492R and 0.25 µl Taq polymerase (5 U/µl) (Merck Bio Sciences, India). Thermocycling conditions consisted of initial one denaturation step at 94°C for 5 min followed by 32 amplification cycles at 94°C for 30 s, 55°C for 40 s, 72°C for 40 s followed by a final extension at 72°C for 5 min. PCR products were analysed by electrophoresis in 2% (w/v) agarose gel in 1x Tris Borate-EDTA buffer and stained with ethidium bromide (5 µg/ ml) and visualized by Alpha imager EP (Alpha Innotech Corporation, USA). PCR products were sequenced to confirm that it has homology identical to the previously reported rDNA sequence of *P. putida* available in NCBI.

The phylogentic analysis of *P. putida* OPf1 was inferred using the Maximum Parsimony method. Tree 1 out of 3 most parsimonious trees (length = 74) is shown. The consistency index was 0.612245, the retention index was 0.707692, and the composite index was 0.523498 (0.433281) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown above the branches (Felsenstein, 1985). The MP tree was obtained using the Tree-Bisection-Regrafting (TBR) algorithm (Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The tree was drawn to scale, with branch lengths computed following the average pathway method (Nei and Kumar, 2000) and expressed in the units of number of changes over the whole sequence. The analysis involved 27 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 406 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

B. subtilis OTPB1 and *T. harzianum* OTPB3 were deposited at National Bureau of Agriculturally Important Microorganisms, Mau, India bearing accession numbers NAIMCC-B-01339 and NAIMCC-F-03065, respectively and *P. putida* OPf1 was deposited at Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India, as accession number MTCC 5824.

Agar plate-based pathogen inhibition assays

Antagonistic effect of *B. subtilis* OTPB1 or *T. harzianum* OTPB3 was evaluated against *P. infestans* PIT 30 by adopting dual culture method (Webber and Hedger, 1986). For inhibition assays by *P. putida* OTPf1, a 5 mm-diameter agar plug of a 7-day-old culture of *P. infestans* PIT 30 was transferred to the center of a plate Rye agar A and incubated at 19 ± 1°C in darkness. Then, 5 µl from an exponentially growing bacterial culture in nutrient broth at OD600 of 0.1 was spotted 1 cm from the edge of the rye agar plate on one side of the pathogen plug. Controls consisted of a 5 mm-diameter agar plug of without *P. putida* OTPf1.

Preparation of bacterial cell suspension

Bacterial inoculum of *B. subtilis* OTPB1 and *P. putida* OPf1 were prepared by harvesting cells from nutrient broth cultures grown at 28 ± 1°C for 48 h followed by centrifugation at 6000 rpm for 15 min. The inoculum was re-suspended in sterile distilled water and then the concentration was adjusted using a Biomate 3 spectrophotometer (Thermo spectronic, USA) to 10^8 cfu/ml (Thompson, 1996; Yan et al., 2002) as confirmed by plating on nutrient agar.

Preparation of spore suspension of *T. harzianum* OTPB3

Spore suspensions of *T. harzianum* OTPB3 were prepared by scraping them from cultures grown on potato dextrose agar plates placed under cool-white fluorescent light with a 12 h alternating light and dark cycle at 25± 1°C for 7 days. Spores were suspended in sterile distilled water and the number of colony forming units (cfu) that developed was assayed on a *Trichoderma* selective medium (Elad et al., 1981) and adjusted the values to 10^8 CFU/ ml.

Preparation of zoospore suspension of *P. infestans* PIT 30

P. infestans PIT 30 (GenBank accession JF834691) was used (Chowdappa et al., 2013b) in the present study. Zoospore suspension was prepared by growing *P. infestans* PIT 30 on Rye agar B medium at 18°C under light (16 h cool white fluorescent light and 8 h dark) for 14 days. Sporangial suspension was obtained from rye agar plates that were gently washed with cold sterile distilled water to liberate sporangia. The sporangial suspension was placed in a refrigerator for 2h to induce zoospore release. Zoospores were separated from sporangia by filtration through a 12-µm mesh filter and diluted to a concentration of $3x10^5$ zoospores /ml.

Test chemicals

Mancozeb was procured from Indofil Chemicals Pvt. Ltd., India and the pre-packed mixture of fenamidone and macozeb was obtained from Bayer Pvt. Ltd., India.

Compatibility between *T. harzianum* OTPB3 and *B. subtilis* OTPB1

In vitro bioassay test was done on potato dextrose agar (Himedia, Mumbai, India) to determine the compatibility of the *T. harzianum* OTPB3 and *B. subtilis* OTPB1. A Petri dish containing PDA medium was spot inoculated with a 48 h-old cell suspension of *B. subtilis* OTPB1 at four different corners on the edge of agar medium. A mycelial plug (4mm diameter, cut from the actively growing edge of a 4 day old mycelial mat on PDA) of *T. harzianum* OTPB3 was placed in centre of the plate and incubated at 25 ± 1°C for 5 days in the dark. Each bioassay was replicated and repeated thrice. The compatibility between *T. harzianum* OTPB3 and *B. subtilis* OTPB1 was also studied by mixing equal ratio (1:1 ml) of cell suspension of *B .subtilis* OTPB1 (10^8cfu ml^{-1}) and conidial suspension of *T. harzianum* OTPB3 (10^8 spores ml^{-1}). The mixture was inoculated into potato dextrose broth and incubated at 25 ± 1°C for 7 days and one loop of culture broth was streaked on potato dextrose agar and incubated at 25 ± 1°C for 3 days in the dark.

Seed treatments

Tomato Cv. Arka vikas seeds were surface sterilized with 1% sodium hypochlorite for 2 min followed by three rinses with sterile distilled water. Ten grams of sterilized tomato seeds were incubated in 50 ml spore suspension (10^8 spores/ml,) of *T. harzianum* OTPB3 or cell suspension (10^8cfu ml^{-1}) of *B.subtilis* OTPB1, amended with 0.2% (w/v) sterile carboxymethyl cellulose (CMC) sticker suspensions at 25°C in a rotary shaker at 80 rpm for 2 h for allowing attachment of bacterial cells or spore suspension or test chemicals to the seed coat. The treated seeds were placed in sterile 90 mm Petri dishes and air-dried on a laminar flow bench for 12 h. For combined inoculation of *Trichoderma* and *Bacillus* isolates, seeds were soaked in a mixture of cell suspension of *B .subtilis* OTPB1 (10^8cfu ml^{-1}) and conidial suspension of *T. harzianum* OTPB3 (10^8 spores ml^{-1}) in ratio of 1:1. Suspension of mancozeb (0.2%) was used. The seeds treated with sterile distilled

water amended with CMC and seeds soaked in distilled water served as controls. Inoculant densities on treated tomato seeds were determined using a dilution plating technique. Five tomato seeds treated with OTPB3, OTPB1 and consortium of OTPB3 and OTPB1 were suspended in 5 ml of 10 mM sterile phosphate buffer (pH 7.0) and sonicated in an ultrasonic bath to release adhering bacteria and *Trichoderma* and then serial dilutions (1/10) were plated on Kings B medium and *Trichoderma* selective medium. Petri dishes were incubated for 5 days at 28°C for bacteria and 7 days at 25°C for *Trichoderma*. The number of cfu per seed was determined at inoculation time (0 h), and 24 h and 48 h from inoculation time (Correa et al., 2009).

Effect of seed treatment on growth promotion under greenhouse conditions

Seeds treated with fresh suspension of microbial consortium and test chemicals along with untreated controls sown separately in pot-trays filled with sterilized coco peat. Seedlings were allowed to grow for 30 days at 25 ± 2ºC under natural light. After 30 days, seedling growth parameters such as root length and shoot lengths, root and shoot weights and leaf area were measured for 1,536 seedlings. Each treatment consisted of four replicates and each replication consisting of 96 plants, thereby making a total of 384 plants per treatment and the experiment was repeated thrice. The germination percentage was calculated on the 14th day after sowing as most of the seeds germinate within this period. Seeds were considered as germinated when their two cotyledonary leaves were visible above the coco peat. About 1,536 seeds (3 independent experiments with four replicates) were scored for determining germination percentage. Seedling vigour index was calculated using the following formula as described by Baki and Anderson (1973) that is seedling vigour index = seedling length (cm) × germination percentage. The data of all 1,536 seedlings were pooled and analyzed after no block effects were noted.

Determination of growth hormones in tomato

IAA and GA_3 levels were determined in the roots of tomato seedlings treated with biocontrol agents, mancozeb and untreated control according to the method of Kelen et al. (2004) with a few modifications. Tomato root samples (10 g) from 30 day old seedlings were macerated in 80% chilled methanol (50 ml) and centrifuged at 4000 rpm for 10 min after leaving the extract overnight at 4°C. The supernatant was evaporated *in vacuo* at 40°C, residue dissolved in water and adjusted to pH 8.0. The alkaline extract was partitioned twice with ethyl acetate and discarded. pH of the aqueous phase of the extracts was adjusted to pH 2.5 using 0.5 N hydrochloric acid. The acidic extract was then partitioned twice with equal volumes of diethyl ether. The diethyl ether portion, after drying over anhydrous Na_2SO_4, was filtered through Whatman No. 1 filter paper, and the ether was removed *in vacuo*. The residue was dissolved in 0.5 ml of 100% methanol for GA_3 and IAA analyses as described below.

High performance liquid chromatography (HPLC) conditions

IAA and GA_3 were assessed by HPLC (Model-Prominence, Make-Schimadzu, Japan) as described by Kelen et al. (2004) with a few modifications. A C_{18} reverse phase column (Synergi, 250 x 4.6 mm, 4 µm, Phenomenex, USA) and photodiode array (PDA) detector (Model SPD-M20A, Schimadzu, Japan) were used in the HPLC system. The solvent system included 70% water at pH 4.0 [adjusted with ortho phosphoric acid (5%)] (B) in acetonitrile (A) at a flow rate of 0.8 ml/min to resolve GA_3 and IAA. The quantification of these phytohormones was carried out at 205 and 220 nm against external standards. The experiment was repeated 12 times with five plants each time.

Induction of systemic resistance

Pot trays containing 30 days-old tomato seedlings treated with different seed treatments were placed in growth chambers (Research and Test Equipment Co,, Bangalore, India). Then, each pot tray containing 96 seedlings were sprayed separately with cell suspensions of *P. putida* OPf1 (10^8cfu ml^{-1}), mancozeb (2 g/l) and pre-packed mixture of fenamidone + mancozeb (3 /l) followed by zoospore suspension containing $3x10^5$ zoospores /ml of *P. infestans* PIT 30 (Chowdappa et al., 2013b). The treated plants were then kept in plant growth chambers and incubated at 18 ± 1ºC with 100% relative humidity under 16 h cool white fluorescent light and 8 h dark (Chowdappa et al., 2013a). The disease incidence was recorded six days after inoculation and rated by estimating the affected percentage leaf area (James, 1971) of all leaves. Percentage of disease severity incidence was calculated using the formula (Amin et al., 2013).

$$\text{Percentage Severity Index} = \frac{\text{Sum of Individual numerical rating}}{\text{Total Number of assessed x Maximum score in scale}} \times 100\%$$

The experiments were repeated thrice. Each experiment consists of 3 pot- trays with 96 plants/tray, totaling 288 plants. Total number of plants used for experiments are 864 seedlings. The data of all the 864 seedlings were pooled and analyzed after no block effects were recorded. The samples for enzyme assay were collected separately during three repetitions.

Sample collection and assay of defense-related proteins

Thirty days old plants were carefully uprooted without causing any damage to root and leaf tissues at intervals of 0, 1, 3, 5, 7, 9 and 11 days after challenge inoculation (Latha et al., 2009). The seedlings from each replication were separately washed in running water, blot dried and homogenized with liquid nitrogen in a pre-chilled mortar and pestle. One gram of sample was homogenized with 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 4°C. The homogenate was centrifuged for 20 min at 10,000 rpm. The supernatant was used as a crude enzyme extract for assaying peroxidase (PO; EC 1.11.1) (Hammerschmidt et al., 1982), polyphenol oxidase (PPO; EC 1.12.18.1) (Mayer et al., 1965) and phenylalanine ammonia lyase (PAL; EC 4.3.1.5) (Dickerson et al., 1984). Enzyme extracted in 0.1 M sodium citrate buffer (pH 5.0) was used for the estimation of β-1,3-glucanase (Pan et al., 1991). Each enzyme assay consisted of eight replications (leaves) and two spectrophotometric readings per replication using a Biomate 3 spectrophotometer (Thermospectronic, USA). Each replication consists of five plants.

Assay of peroxidase (EC 1.11.1)

The assay was carried out as described by Hammerschmidt et al. (1982). The reaction mixture consisted of 1.5 ml of 0.05 pyrogallol, 0.5 ml enzyme extract and 0.5 ml of H_2O_2 and incubated at 28±1°C. The changes in absorbance were measured at 42 0nm at 30 s interval for 3 min. The enzyme activity was expressed as changes in absorbance of the reaction mixture min^{-1}g^{-1} on fresh weight source.

Assay of polyphenol oxidase (EC 1.12.18.1)

Enzyme assay was performed as described by (Mayer et al., 1965).

200 µl of enzyme extract was added with 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5). Reaction was initiated by adding 200 µl of 0.01catechol. Changes in absorbance of the reaction mixture were expressed in $min^{-1}g^{-1}$ on fresh weight source.

Assay of phenylalanine ammonia lyase (EC 4.3.1.5)

Enzyme assay was performed as described by Dickerson et al. (1984). Reaction mixture containing 100 µl of enzyme with 500 µl of 50 mM Tris HCL (pH 8.8) and 600 µl of 1 mM L- phenylalanine were incubated for 60 min at 25°C. The reaction was arrested by adding 2 N HCl. Meanwhile 1.5 ml of toluene was added, mixed in vortex for 30 s, centrifuged at 10,000 rpm at 4°C for 5 min. Toluene portion with trans-cinnamic acid was separated and toluene phase was read at 290 nm against toluene as blank. A standard curve was plotted using cinnamic acid solution in toluene at described concentrations.

Assay of β-1, 3-glucanase (EC 3.2.1.39)

Assay was carried out as using laminarin dinitrosalicylic acid method as described by Pan et al. (1991). The reaction mixture consisted of 62.5 µl of 4% laminarin and 62.5 µl of enzyme extract. The assay was carried out at 40°C for 10 min. The reaction was terminated by adding 375 µl of dinitrosalicylic acid and heating for 5 min in hot water bath, mixed well and measured absorbance at 500nm. The activity was expressed as µg of glucose released units/mg of protein.

Protein estimation

Protein contents of the extract for all enzymes were estimated following the method of Bradford (1976) using bovine serum albumin (BSA) (Sigma, USA) as standard.

Native polyacrylamide gel electrophoresis analysis

The isoform profiles of PPO were separated by discontinuous native polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). The protein extract was prepared by homogenizing 1 g of leaf sample in 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged at 18,000 rpm for 20 min at 4°C. After the protein content was determined (Bradford, 1976), the samples (50 µg protein) were loaded onto 8% polyacrylamide gels (Sigma, USA). After electrophoresis, PPO isoform profiles were assessed by equilibrating gels for 30 min in 0.1% p-phenylene diamine, followed by addition of 10 mM catechol in the same buffer (Jayaraman et al., 1987).

Statistical analysis

All data were statistically analyzed using one way analysis of variance (ANOVA) to identify the origin of significance and followed up with a Fishers test to separate means and treatments using Graphpad Prism V.5.00 for windows (Graph pad software, San Diego, California, USA). Means were compared between treatments by least significant difference (LSD) at the 1% level ($p<0.01$). Percentage data were arcsine-transformed before analysis according to $y = \arcsin [\sqrt{(x/100)}]$.

RESULTS

Identification of *Pseudomonas putida* OPf1

PCR amplication of the 16S rDNA gene amplified from the genomic DNA of *P. putida* OPf1 yielded fragment of 1464 bp. Blast search of the *P. putida* OPf116S rDNA gene sequence revealed that it had 98% similarity to the 16S rDNA gene sequences of *P. putida* strains in NCBI (Figure 1). A phylogenetic tree generated using 16S rDNA gene sequences showed that *P. putida* OPf1 was closely related to *P. putida* (Figure 1). The OPf1 was identified as *P. putida,* based on the sequence analyses of 16S rDNA gene. The 16S rDNA sequence of OPf1 was deposited in NCBI (www.ncbi.nlm.nih.gov/) with accession no. KC964109.

In vitro evaluation of antagonists

The *P. putida* OPf1 significantly reduced mycelial growth of *P. infestans* by 72.9% when evaluated under *in vitro* conditions (Table 1).

Compatibility between *T. harzianum* OTPB3 and *B. subtilis* OTPB1

When one loop of culture broth streaked on potato dextrose agar, both *B. subtilis* OTPB1 and *T. harzianum* exhibited growth on PDA without any antagonistic activity after 72 h of incubation (Figure 2). They also did not exhibit inhibitory effects on each other when spot inoculated on PDA. The number of colony forming units (cfu) recovered from treated seed at different time intervals after inoculation (Table 2) showed that OTPB3, OTPB1 and microbial consortium were effectively colonized tomato seeds. No differences were observed in colony forming units, irrespective of treatment and remained unaffected up to 48 h of post inoculation (Table 2). Thus, the isolates OTPB3 and OTPB1 were compatible and can be utilized for seed coating formulation (Table 2).

Growth parameters

Tomato seeds treated with a mixture of *B. subtilis* OTPB1 and *T. harzianum* (OTPB3) or singly with OTPB1, OTPB3 and *P. putida* OPf1 exhibited increase ($p<0.01$) in seedling growth parameters (Table 3) significantly compared to mancozeb (0.2%) and untreated control. The consortium enhanced root and shoot lengths, leaf area, fresh weight of shoots and roots by 56.3, 40.9, 34.0, 50.2 and 56.9% respectively as compared to the control seedlings (Table 3). The data also indicated that the microbial consortium stimulated better growth than

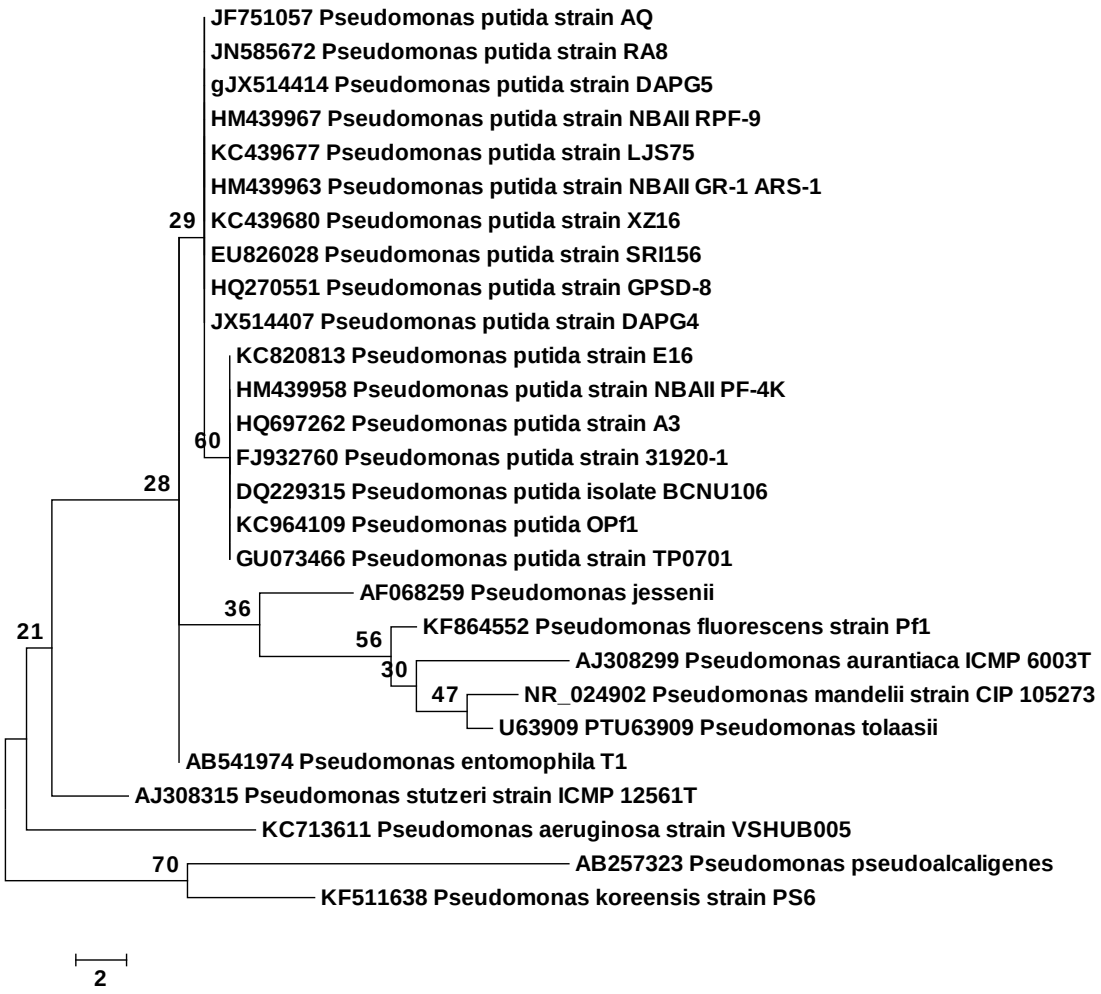

Figure 1. Phylogenetic tree of the *Pseudomonas putida* OPf1 based on the 16s rDNA gene sequences.

Table 1. *In vitro* inhibition of and *P. infestans* (OTA 30) by *P. putida* (OPf1)[A].

Isolate	Pathogen	Pathogen growth (mm)	
***P. putida* (OPf1)**	*P. infestans* (PIT30)	21.0 ± 1.0	(72.9)
Control	*P. infestans* (PIT30)	77.7 ± 0.5	

Values in parentheses indicate percent inhibition of pathogen growth over control. Percentage of inhibition was calculated based on data collected after seven days of inoculation. Inhibition percentage defined as [C-T/C](100)], where C is the colony diameter of pathogen on control plate and T is the colony diameter of pathogen against test antagonist(mm). Percentage data were arcsin-transformed before analysis according to y = arcsin [sqr. (/100)]. Data are the means and standard deviation of nine independent experiments. Each experiment contained three replicates. Each replicate contained six Petri plates.[A] *Phytophthora infestans* inhibition assay on rye A agar 19 ± 1°C were performed. The radial growth of the pathogens were measured after every 24 h till the fungus reached the perimeter of the control plate (up to 7 days).

other treatments including treatments with OTPB1, OTPB3 and *P. putida* OPf1 separately. Further studies were restricted to consortium only as they stimulated higher growth and the data on stand-alone treatments of OTPB1, OTPB3 was published earlier (Chowdappa et al., 2013b).

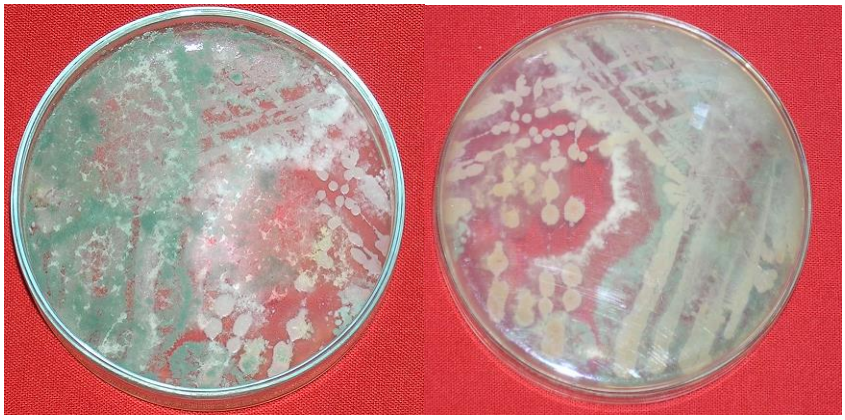

Figure 2. Compatibility between *T. harzianum* OTPB3 and *B. subtilis* OTPB1. Both *B. subtilis* OTPB1 and *T. harzianum* exhibited growth on PDA without any antagonistic activity after 72 h of incubation. One loop of culture broth inoculated with *T. harzianum* OTPB3 and *B. subtilis* OTPB1 was streaked on potato dextrose agar, both *T. harzianum* OTPB3 and *B. subtilis* OTPB1, exhibited growth on PDA without any antagonistic activity.

Table 2. Viable inoculum densities per tomato seed treated with biocontrol agents OTPB1 and OTPB3[A].

Treatments [B]	0 h	24 h	48 h
OTPB3	$5.8x10^4$a	$5.6x10^4$b	$5.8x10^4$c
OTPB1	$5.3x10^4$a	$5.3x10^4$b	$5.6x10^4$c
OTPB3+OTPB1 (OTPB1)	$5.6x10^4$a	$5.3x10^4$b	$5.3x10^4$c
OTPB3+OTPB1 (OTPB3)	$5.6x10^4$a	$5.7x10^4$b	$5.2x10^4$c
CD1%	0.9	1.2	1.3

[A]Values are mean of five independent experiments. Each experiment consists of five seeds. For each row values are followed by a different lower case letter indicates significantly different at $p < 0.01$ for each of pair of treatment according to Fishers LSD test. [B]Five tomato seeds var. Arka vikas, treated with OTPB3, OTPB1 and Consortia with OTPB3 and OTPB1 were suspended in 5ml of 10mM sterile phosphate buffer (pH 7.0) and sonicated in an ultrasonic bath to release adhering bacteria and *Trichoderma* and then serial dilutions (1/10) were plated on Kings B medium for bacteria, Trichoderma selective media for *Trichoderma* and Potato dextrose agar for microbial consortia. Petri dishes were incubated for 5 days at 28^{O}C for bacteria and 7days for *Trichoderma*. The number of cfu per seed was determined at inoculation time (0 h), 24 h and 48 h from inoculation time (Correa et al., 2009).

Growth hormones in tomato

The endogenous levels of IAA and GA_3 in roots of tomato seedlings treated with microbial consortium of *T. harzianum* (OTPB3) and *B. subtilis* (OTPB1) were significantly higher (*P < 0.01*) compared to treatment with *P. putida* OPf1 and mancozeb and untreated control (Table 4). The IAA and GA_3 levels were higher by 71.1 and 78.8%, respectively in seedlings treated with microbial consortium as compared to untreated control, while *P. putida* (OPf1) treated seedlings showed an increase of IAA by 44.7% and GA*3* by 60.7%.

Late blight incidence under controlled conditions in growth chamber

Seed treatment with OTPB3 and OTPB1 combination, OPf1 strain and mancozeb as chemical check coupled with foliar sprays of OPf1 and pre-packed mixture of fenamidone - mancozeb were evaluated for their efficiency against *P. infestans* under pot culture conditions in growth chamber (Table 5). Seed treatment with OTPB3+OTPB1 followed by foliar spray of OPf1 significantly reduced late blight incidence by 73.1% compared to untreated controls. The results showed that disease reduction with OTPB3+OTPB1+ OPf1 mixture was on par with the fungicide check (mancozeb + fenamidone – mancozeb), which also recorded 72.8% reduction in late blight incidence. The combinations of OPf1+ fenamidone – mancozeb, OTPB3+OTPB1+ fenamidone – mancozeb, OTPB3+OTPB1+ mancozeb also caused reduction in late blight incidence similar to OTPB3+OTPB1+ OPf1 combination. Seed treatment alone with OTPB3+OTPB1 combination showed lower incidence of late blight (38.4%) compared to OPf1 (61.2%) and mancozeb (51.4%).

Response of defense-related proteins

PO, PPO, PAL and β-1,3-glucanase activities were measured in leaves from *P. infestans* inoculated and OTPB3 + OTPB1 + OPf1, OTPB3 + OTPB1 + mancozeb, OTPB3 + OTPB1 + fenamidone – mancozeb, OPf1+ fenamidone – mancozeb and OTPB3 + OTPB1, mancozeb, and OPf1 pre-treated tomato plants. These treatments differed in their ability to stimulate PO, PPO, PAL and β-1,3- glucanase activities in tomato plants inoculated with *P.infestans*. The data showed that tomato plants treated with OTPB3 + OTPB1 + OPf1 mixture exhibited higher

Table 3. Effect of seed treatment of fresh suspensions on growth of tomato seedlings[A].

Treatment[B]	Root Length(cm)	Shoot length (cm)	Seedling vigour index[C]	Root weight (g)	Shoot weight (g)	Leaf area(cm^2)
OTPB3	14.4±2.2(41.1)[b]	20.5±2.4(42.0)[b]	3434.0±348.1(45.7)[b]	1.3±0.3(52.9)[b]	2.0±0.4(63.2)[ab]	7.1±1.3(46.6)[b]
OTPB1	11.7±2.7(27.8)[c]	22.3±3.1(35.9)[c]	2935.9±271.3(36.4)[c]	1.0±0.1(26.9)[b]	1.8±0.3(60.4)[b]	6.3±1.8(40.3)[c]
OTPB3+OTPB1	16.5±2.3(56.3)[a]	23.5±2.2(40.9)[a]	3708.9±178.2(53.5)[a]	0.3±0.05(56.9)[a]	2.4± 0.2(50.2)[a]	8.8±1.1 (34.0)[a]
OPf1	11.0±1.4 (34.5)[c]	18.4±1.8(37.9)[c]	2877.7±118.5(40.1)[c]	0.2±0.03(38.2)[c]	1.8±0.3 (46.2)[c]	6.2±1.2 (19.2)[c]
Mancozeb (0.2%)	7.3±1.1 (1.5)[d]	16.6±1.4(16.4)[d]	1952.7±114.3(11.7)[d]	0.1± 0.05(8.5)[d]	1.6± 0.2(23.2)[d]	6.2±1.4 (5.4)[d]
Control CMC	7.8±1.3d	14.0±1.6[d]	1785.8±145.2[d]	0.1±0.05[d]	1.3±0.1[d]	6.0±1.1[d]
Control	7.2±1.2[d]	13.8±1.8[d]	1723.3±138.6[d]	0.1±0.025[d]	1.2±0.2[d]	5.8±1.4[d]
CD 1%	1.3	0.9	127.1	0.04	0.3	0.5

[A] Values are mean of 3 independent experiments ± standard deviation. Each experiment consists of 4 pot trays with 96 plants/tray, totaling 384 plants. Total number of plants used for experiments are 1536 seedlings. Seedling growth parameters like root length, shoot length, root fresh weight, shoot fresh weight and leaf area were determined for 1536 seedlings 30 days after sowing. Values in parentheses indicates percentage increase over control. For each row values followed by a different lower case letter are significantly different at $p < 0.01$, according to Fishers LSD test. [B]Bacterial isolate *B. subtilis* OTPB1 (10^8) and one isolate *Trichoderma harzianum* OTPB3 suspension (10^8), *P. putida* OPf1 suspension (10^8) and consortium of OTPB1 (10^8) and OTPB3 (10^8) and 0.25% suspension of mancozeb were used as fresh suspension for seed treatment and each treated tomato seed var. Arka vikas was placed in each cavity of pot trays containing sterilized cocpeat. Seed receiving only sterile distilled water and CMC for seed treatment served as untreated control and growth parameters were recorded after 30 days of sowing. [C]Seedling vigour index = seedling length (cm) x germination percentage .Vigor indices were calculated after 4 weeks.

Table 4. Ability of Biocontrol agents to induce growth hormones in tomato roots[A].

Treatments[B]	IAA (nmol/g)[B]	GA3 (nmol/g)[B]
OTPB1+OTPB3	35.8±0.8(71.1)a	10.4±0.4(78.8)a
OPf1	18.7±0.9(44.7)b	5.6±0.2(60.7)b
Mancozeb	11.01±0.8(6.08)c	2.2±0.2c
Control	10.34±0.2ce	2.2±0.3c
CD1%	5.7	0.5

[A]Values are mean of six plants ± standard deviation. Five plants each were drawn from 12 independent experiments.Values in parentheses indicate percentage increase over control. For each row values followed by a different lower case letter are significantly different at $p < 0.01$, according to Fishers LSD test. [B]Indole-3-Acetic acid (IAA) and Gibberlic acid (GA3)levels were determined in the roots of the tomato seedlings treated with bio-control agents and untreated control using HPLC method by macerating tomato root samples (10 g) from 30 day old seedlings in 80% chilled methanol (50 ml). The quantification of these phytohormones was carried out at 205 and 220 nm using external standards.

defense enzyme activities against *P. infestans* compared to other treatments (Figure 3). The enzyme activities were increased after 3 days and reached to a maximum after 5 days of pathogen inoculation and decreased, thereafter. However, the enzyme activities in tomato plants treated with a combination of OTPB3+OTPB1+ OPf1 remained high, up to 11 days after inoculation as compared to all other treatments. In contrast, the increased activities of enzymes were observed only up to the seventh day of *P. infestans* inoculation in other treatments and, thereafter, a drastic decline was recorded. Control plants or inoculation with pathogen alone did not exhibit any noticeable changes in the activities of the enzyme (Figure 3).

Native polyacrylamide gel electrophoresis analysis of PPO

An analysis of PPO extract from tomato plants treated with OTPB3+OTPB1+ OPf1 combination and inoculated with *P. infestans* by native PAGE exhibited three isoforms PPO1, PPO2 and PPO3, whereas in other treatments only two isoforms PPO1 and PPO2 were observed with very low intensity and were absent in untreated plants (Figure 4). Further, the expression of isoform banding patterns in plants treated with OTPB3+OTPB1+ OPf1 was more intense compared to other treatments and untreated control.

DISCUSSION

The results from agar plate and seed assays indicated that the isolates of *T. harzianum* (OTPB3) and *B. subtilis* (OTPB1) were compatible. Previous studies showed that biocontrol agents should be compatible when combined in order to obtain desired and consistent plant growth promotion and disease suppression (Janisiewicz and Bors 1995; Raaijmakers et al., 1995; Janisiewicz 1996; Li and Alexander, 1988). Many earlier reports also illustrated

Table 5. Effect of seed treatments with fresh suspensions of OTPB3+OTPB1, OPf1 and fungicides and foliar sprays of fungicides on late blight incidence of tomato under controlled conditions in growth chamber [A].

Treatments[B]	Late blight incidence (%)[C]
Pathogen spray alone	76.6±2.3[e]
OTPB3+OTPB1*	38.4±2.4 (49.9)[c]
Mancozeb*	51.4±3.2 (32.9)[b]
OPf1*	61.2±4.1(20.1)[d]
OTPB3+OTPB1+OPf1**	20.6±2.4(73.1)[a]
OTPB3+OTPB1+ Fenamidone + Mancozeb**	20.0±2.4(73.9)[a]
Mancozeb + Fenamidone + Mancozeb**	20.6±2.2(72.8)[a]
Mancozeb + OPf1**	25.4±2.1(66.8)[a]
OPf1+ Fenamidone + Mancozeb**	20.5±3.1(68.0)[a]
OPf1+OPf1**	48.2±3.5(37.1)[c]
Control CMC	71.4±3.4(6.8)[e]
CD 5%`	14.6

*Seed treatment; **Seed treatment with foliar spray. [A]Values are mean of 3 independent experiments ± standard deviation. Each experiment consists of 3 pot trays with 96 plants/tray, totaling 288 plants. Total number of plants used for experiments are 864 seedlings. Percentage of disease severity index was estimated after initiation of symptom, *i.e.,* 72 hrs of pathogen spray. Values in parentheses indicate percent inhibition of pathogen growth over control. Percentage of inhibition was calculated based on data collected after seven days of inoculation. Inhibition percentage defined as [C-T/C](100)], where C is the late blight incidence of control plant and T is the late blight incidence of treated. Percentage data were arcsin-transformed before analysis according to y = arcsin [sqr. (_/100)]. For each row values followed by a different lower case letter are significantly different at $p < 0.05$, according to Fishers LSD test. [B]Pot trays containing tomato seedlings of 30 days old treated with different seed treatments were placed in growth chambers were sprayed with different foliar treatments which includes *P. putida* OPf1, Mancozeb and Famaxodine + Mancozeb followed with spray of *P.infestans* PIT 30 spore suspension and plants were incubated in 100% relative humidity (RH) and maintained at 25 ^{0}C at day and 20°C at night, with a 12-h photoperiod (Yan et al., 2002). Six days after inoculation with the pathogen, disease was rated by estimating the affected percentage leaf area (James, 1971) of all leaves and percentage of disease severity incidence was calculated using the formula (Amin et al., 2013).

$$\text{[C]Percentage Severity Index} = \frac{\text{Sum of Individual numerical rating}}{\text{Total Number of assessed x Maximum score in scale}} \times 100$$

that disease suppression can be increased by utilizing combinations of biological control agents and plant growth promoting rhizobacteria (PGPR) and their combined effects are pronounced in improving crop yields and enhancing nutrient uptake by plants (Alagawadi and Gaur, 1988; Alagawadi and Gaur, 1992, Jisha and Alagawadi, 1996; Guetsky et al., 2002; (van Peer et al., 1991; Duffy et al., 1996; de Boer et al., 1999; Nandakumar et al., 2001; Domenech et al., 2006; Saravanakumar et al., 2007; Thilagavathi et al., 2007; Ganeshmoorthi et al., 2008; Latha et al., 2009) over single organism inoculations. Meanwhile Yobo et al. (2009) demonstrated that *Trichoderma* and *Bacillus* combinations were better than the *Trichoderma* isolated and *Bacillus* isolates used alone. They reported that there was potential in using mixtures of *Trichoderma* and *Bacillus* for improving plant growth and disease control. Earlier studies also demonstrated that the mixtures of *T. harzianum* and *B. subtilis* may not affect each other *in vivo* due to spatial separation on the roots or production of antimicrobial

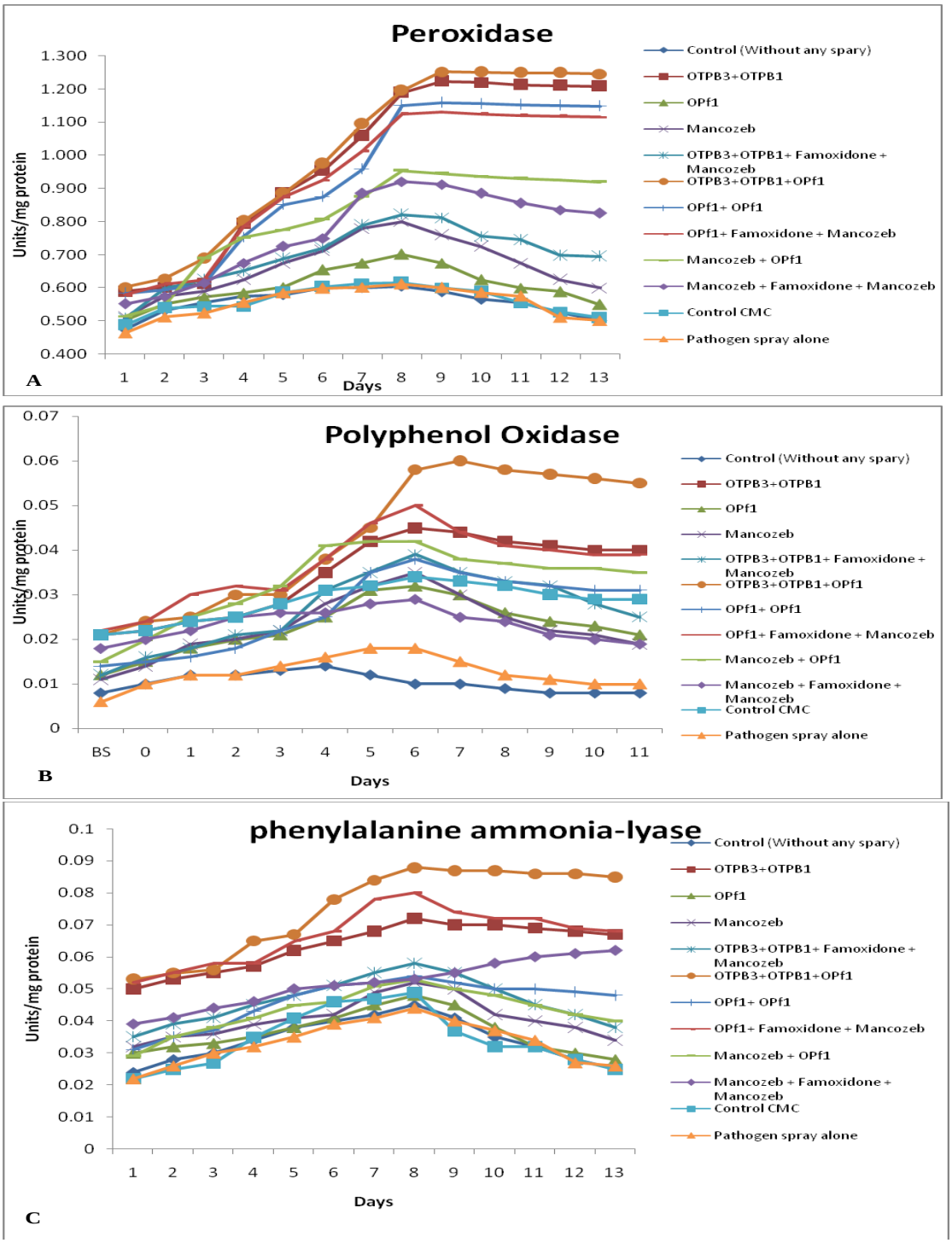

Figure 3. Induction of peroxidase (A), polyphenol oxidase (B), phenylalanine ammonia-lyase (C) and β-1,3-glucanase (D) activities in tomato plants treated with bio-control agents and fungicides extract against *P. infestans.* Thirty day old plants root and leaf tissues were collected at different day intervals viz., 0, 1, 3, 5, 7, 9 and 11 days after challenge inoculation (Latha, 2009). Four fresh seedlings were selected from each replication and they were washed in running water, blot dried and homogenized. One gram of sample was homogenized with 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 4°C. The homogenate was centrifuged for 20 min at 10,000 rpm. The supernatant was used as a crude enzyme extract for assaying peroxidase (PO; EC 1.11.1) (Hammerschmidt et al., 1982), polyphenol oxidase (PPO; EC 1.12.18.1) (Mayer et al., 1965) and phenylalanine ammonia lyase (PAL; EC 4.3.1.5) (Dickerson et al., 1984). Enzyme extracted in 0.1 M sodium citrate buffer (pH 5.0) was used for the estimation of β-1,3-glucanase (Pan et al., 1991) . Each of the enzyme assays were repeated three times. BS, before pathogen spray.

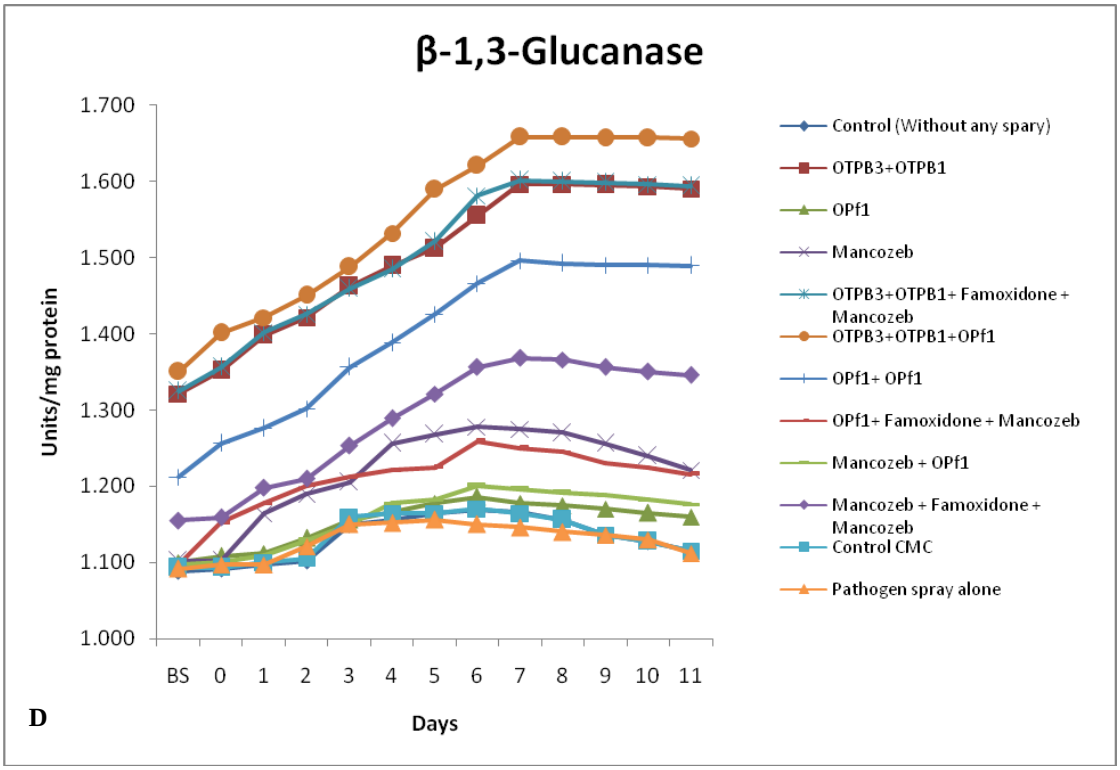

Figure 3. Cntd.

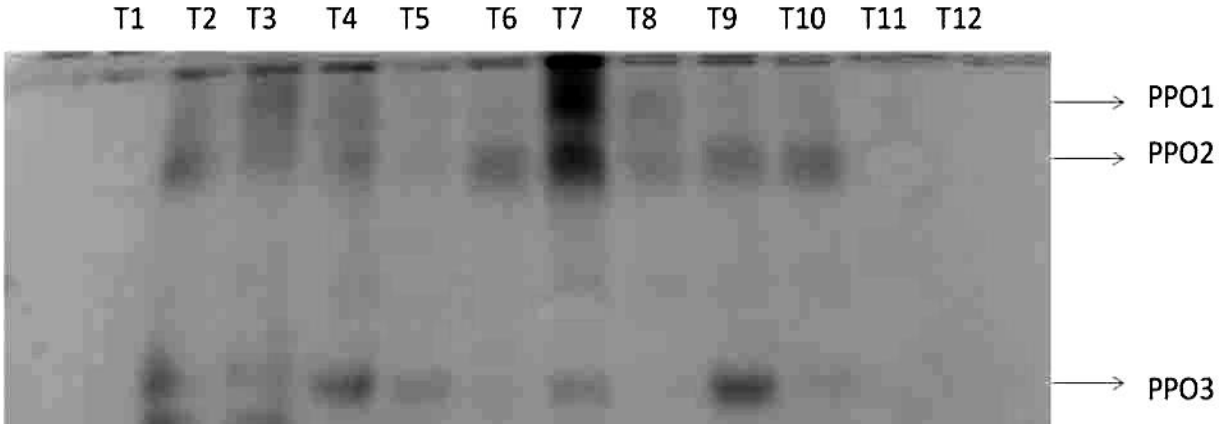

Figure 4. Native polyacrylamide gel electrophoresis analysis of polyphenol oxidase in tomato plants treated with bio-control agents and fungicides against *P. infestans*. Lane, T1: Control (without any spray); T2, OTPB3+OTPB1; T3, Mancozeb; T4, OPf1; T5, Mancozeb + Fenamidone + Mancozeb; T6, Mancozeb + OPf1; T7, OTPB3+OTPB1+OPf1; T8, OTPB3+OTPB1+ Fenamidone + Mancozeb; T9, OPf1+ Fenamidone + Mancozeb; T10, OPf1+OPf1; T11, Control CMC; T12, Pathogen alone spray.

compounds *in vitro* in the stationary phase (Fukui et al., 1994; Duffy et al., 1996). Compatible combinations of biocontrol agents might be useful to deal with multiple diseases or multiple infection sites of a disease or wide range of environmental conditions (Fukui et al., 1994) as single isolate may not work in different situations or against different pathogens. Most cases of naturally occurring biological control results from mixtures of antagonists rather that from high population of a single antagonist (Bin et al., 1991). Accordingly, application of a

mixture of pioneered biocontrol agents would further closely imitate the natural condition and might broaden the spectrum of biocontrol activity, improve the efficiency and consistency of biological control (Mishra et al., 2011). Direct interactions taking place among members of dissimilar microbial types often result in the promotion of key processes benefiting plant growth and health. Syntrophic relationships between different organisms have been demonstrated in several microbial ecosystems. Hence combinations of microorganisms that interact synergistically are currently being devised, which yield better and quick results (Bashan, 1998). Hence microbial consortium was suggested for plant growth promotion and disease suppression (Seneviratne, 2003). However, information pertaining to combined inoculations of *Trichoderma* and *Bacillus* species on plant growth and especially on disease control appears to be very sparse, even though both *Bacillus* and *Trichoderma* species are well known for their biological control and plant growth promoting properties (Yobo et al., 2009).

Tomato seeds coated with fresh suspensions of microbial mixture containing *T. harzianum* (OTPB3) and *B. subtilis* (OTPB1) resulted in significant increase in growth parameters in comparison with *P. putida* OPf1 and mancozeb treatments and untreated control. Many strains of *Trichoderma* spp., *Bacillus* spp. and *Pseudomonas* spp. were reported as potential plant growth promoters and disease resistance inducers in a range of crops (Schneider and Ullrich, 1994; Raupach and Kloepper, 1998; Nandakumar et al., 2001; Ramamoorthy et al., 2002; Harman et al., 2004; Kleifeld and Chet, 1992; MacKenzie et al., 1995; Windham et al., 1986; Yedidia et al., 1999; Chithrashree et al., 2011; Chowdappa et al., 2013b). Choure et al. (2012) demonstrated that use of microbial consortia promoted early growth in *Cajanus cajan,* compared to individual strains of *S. fredii* KCC5, *P. fluorescens* LPK2 and *Azotobacter chroococcum* AZK2. Senthilraja et al. (2010) also reported that *B. bassiana* and *P. fluorescens* formulation has effectively decreased the collar rot and increased yield in groundnut production.

The significant increase in growth parameters of tomato was possible due to higher production of IAA and GA_3 in roots of tomato seedlings raised from seeds coated with *T. harzianum* (OTPB3) and *B. subtilis* (OTPB1) consortium. The enhancement of IAA and GA_3 levels is one of the mechanisms by which biocontrol organisms can enhance shoot and root growth and leaf area in tomato plants. IAA plays a vital role in initiation and elongation of lateral and adventitious roots and also influence shoot development (Hedden and Thomas, 2006). GA_3 in combination with auxins promotes axial part elongation (Srivastava, 2002). IAA stimulates cell elongation or cell division by reducing the effect of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity and promotes root growth. ACC is a known inhibitor of root growth and several bacteria produce ACC-deaminase (Jacobson et al., 1994). Many studies demonstrated that certain bacteria and fungi promote plant growth directly through increased nutrition uptake excited by growth regulators (Idris et al., 2007; Gravel et al., 2007; Harman, 2011; Shoresh et al., 2010; Kloepper et al., 2004; Chen et al., 2007; Chowdappa et al., 2013b). They also colonize plant roots, suppress many soil borne fungal pathogens and also stimulate growth and crop yield (Idris et al., 2007).

Accumulation of enzymes such as peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and β-1, 3-glucanase were significantly higher in tomato seedlings treated with OTPB3+OTPB1 consortium followed by *P. putida* OPf1 foliar spray after challenge inoculation with *P.infestans* as compared to other treatments including fungicidal check, mancozeb, fenamidone – mancozeb, and untreated control and is presumably responsible for the reduction of late blight disease index in plants challenged with *P. infestans.* Enhanced activities of the enzymes related to defense in the PGP microbes treated tomato plants may play a role in suppression of pathogen interference in the host eventually preventing development of disease. Several studies have demonstrated that enhancement of PO, PPO, PAL and β-1, 3-glucanase activities were responsible for fungal disease suppression in plants treated with *T. harzianum* (Jayalakshmi et al., 2009; Houssien et al., 2010) or *B. subtilis* (Nakkeeran et al., 2006; Thilagavathi et al., 2007; Latha et al., 2009; Chitrashree et al., 2011) or *Pseudomonas* spp (Latha et al., 2009, Sundaramoorthy et al., 2012).

In the present study, enzyme activities were initiated 72 h after pathogen inoculation and were maximum on 5^{th} day in all treatments. Plants treated with OTPB3+OTPB1 consortia followed by OPf1 foliar spray exhibited maximum activities of the defense enzymes during the initial stage of pathogen attack and persisted up to 11^{th} day after pathogen inoculation, which may be the cause of reduction in late blight disease incidence. Similar kind of responses were reported in many host-pathogen interactions (Dalisay and Kuc, 1995; Chen et al., 2007; Ramamoorthy et al., 2002; Rajendran and Samiyappan, 2008). Increased accumulation of both PO and PAL plays an important role in biosynthesis of secondary metabolites and phytoalexins and attributed their role in disease resistance (Daayf et al., 1997; Ryals et al., 1996; Kosuge, 1969). Increased activity of PO and PAL was reported in tomato treated with *P. fluorescens* infected by *Fusarium oxysporum* (Ramamoorthy et al., 2002), PO, PPO and PAL activity in rice, treated with *B. pumilus* SE34 and *B. subtilis* GBO3 after challenge inoculation with *Xanthomonas oryzae pv. oryzae* (Chithrashree et al., 2011). β-1-3-glucanase have the ability to hydrolyze β-1-3-glucan, a major component of cell wall of Stramenopile fungus like, *P.infestans* leading to direct the inhibition of growth of pathogen (Karthikeyan et al., 2005). Umamaheswari et al. (2009) reported that watermelon plants pre-treated with bio-agents showed enhanced PAL, PO, PPO, β-1-3-glucanase activities upon challenge inoculation with *Alternaria alternata*.

The present study is clearly demonstrated better ability of the 'synthetic microbial consortium' of *T. harzianum* (OTPB3) and *B. subtilis* (OTPB1) to promote plant growth and induce systemic resistance against *P. infestans* in tomato than those of seed treatments with mancozeb and stand-alone treatments of OTPB3 and OTPB1. Thus, development of seed coating formulation with the microbial consortium of OTPB3 and OTPB1 is crucial to raise healthy tomato seedlings as *P.infestans* is a soil/seed borne pathogen (Wangsomboondee and Ristaino, 2002). In addition to seed and soil borne inoculums, airborne inoculum is also vital to late blight outbreaks under congenial tropical and subtropical conditions. In practice, protective foliar fungicidal applications at weekly intervals are used to effectively control the late blight disease. Thus, seeds treated with consortium of OTPB3 and OTPB1 followed by OPf1 foliar spray showed persistence of higher activities of the defense enzymes up to 11th day after pathogen inoculation leading to reduction in late blight disease incidence. This synthetic microbial consortium has the ability to protect plants from soil/seed/air borne inoculums. As most of the vegetable growers in India purchase tomato seedlings from commercial vegetable nurseries grown in pot trays using coco peat, movement of the *P. infestans* through seedlings is very high and this can be contained through seed treatments. Systemic resistance can be extended in field by foliar spray of *P. putida* OPf1 comparable with results of fungicide check fenamidone-mancozeb .

Therefore, in comparison with our previous work, where basal application of isolates of *T. harzianum* OTPB3 or *B. subtilis* OPTB1 individually promoted growth and induced systemic resistance against early and late blight of tomato, and in present paper, the effects of growth promotion and induction of systemic resistance are more in the tomato seedlings when seeds treated with consortium of OTPB3 and OTPB1 followed by OPF1 spray..

We, therefore, suggest that a combination of OTPB3 and OTPB1 can be effectively used for development of seed coating formulations to produce disease free and quality tomato seedlings and *P. putida* OPf1 as foliar spray for effective management of late blight disease.

However, this technology 'synthetic microbial consortia' needs to be validated further under field conditions at multi-locations before any recommendations are made.

Conflict of interests

The authors did not declare any conflict of interest.

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Microbiological assessment of traditional smoked silver catfish (*Chrysichthys nigrodigitatus*)

Adeyeye, S. A. O.[1]*, Oyewole, O. B[1]., Obadina, A. O.[1] and Omemu, A. M.[2]

[1]Department of Food Science and Technology, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria.
[2]Department of Hospitality and Tourism Management, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria.

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Smoked fish is relished food item in many dishes in Nigeria. Traditional smoked silver catfish (*Chrysichthys nigrodigitatus*) that floods the smoked fish market of the Lagos State of Nigeria are not microbiologically shelf-stable; hence, the need for a study on their microbiological quality and safety. 'Fresh silver catfish' (100 samples) and smoked silver catfish (100 samples) were collected from 20 different processing centres and the fresh samples were smoked with convectional smoke kiln as control and microbiological analyses were conducted. Each batch was assessed for: total viable count (TVC), fungal count (FC), *Listeria monocytogenes* (LM) count, *Staphylococcus aureus* (SA) count, *Salmonella* Paratyphi (SP) count and presence or absence of *Escherichia coli* (EC). The results obtained showed significant variations ($p<0.05$) for all the microbial counts of the smoked fish samples. The mean TVC range of 6.6×10^6 - 8.8×10^8 cfu/g was recorded for fresh silver catfish samples and 2.0×10^4 - 8.6×10^4 and 1.0×10^3 - 5.4×10^3 cfu/g for samples of smoked silver catfish. 5.0×10^3 - 6.4×10^3 cfu/g was recorded for fresh silver catfish samples and from 5.6×10^2 - 60.4×10^2 cfu/g and 1.0×10^2 - 3.5×10^2 cfu/g for samples of smoked silver catfish (SA). Mean (LM) of fresh silver catfish samples was 1.8×10^2 - 2.5×10^2 cfu/g and 1.3×10^1 - 13.2×10^1 cfu/g for samples of smoked silver catfish. Mean (FC) of smoked silver catfish from local drum kiln was 4.1×10^1 - 8.2×10^1 cfu/g. The samples of smoked silver catfish using conventional smoke kiln showed no count for *L. monocytogenes, S.* Paratyphi *and E. coli*.

Key words: Silver catfish, smoking, traditional, *Staphylococcal*, *Listeria monocytogenes.*

INTRODUCTION

Fish is an important dietary component of people all around the world and represents a relatively cheap and accessible source of high quality protein for poorer households (Ikutegbe and Sikoki, 2014). In West Africa, fish has been reported to provide 40–70% of the protein intake of the population (Béné and Heck, 2005; Ikutegbe and Sikoki, 2014) and is a critical source of dietary protein that is not readily available in the carbohydrate-based staple foods of the population. Depending on consumer preference, there are several forms in which fish can be consumed; fresh, dried, frozen, fermented, brined, etc. In a study by Mafimisebi (2012), it was discovered that majority of the Nigerian people reported a preference for fresh fish; however limitations such as the

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

low keeping quality of the fish after harvest and the distances between fishing grounds and marketing outlets make this very difficult. This results in a higher reported consumption of smoke-dried fish, which has a longer shelf-life (Mafimisebi, 2012). In Nigeria, fish has an edge over meat because it is cheaper and relatively more abundant (Eyo, 2001) and constitutes about 40% of the animal protein intake (Eyo, 2001; Abolagba and Melle, 2008).

Because fish is highly perishable, a considerable effort has been directed to extend its shelf-life using preservation and processing techniques, such as refrigeration, freezing, canning, smoking, salting and drying (Nwachukwu and Madubuko, 2013). Besides this, some of these techniques can also be used to enhance the value of fish, such as smoked fish.

Smoked fish is relished food item in many dishes in Nigeria. The technique has developed to a point where once common food has become a delicacy and there is need for corresponding concern for safety issues in smoked fish consumption (Riches, 2012). Da Silva et al. (2008) examined the microbial safety and quality of smoked blue catfish (*Ictalurus furcatus*) steaks treated with antimicrobials and antioxidants during 6 weeks ambient storage. Fafioye et al. (2002) studied the fungal infestation of five traditionally smoked dried freshwater fish in Ago-Iwoye, Nigeria, and isolated and identified eleven different fungal species of which *Aspergillus flavus* was the most frequently encountered fungi on the fish species. Adebayo-Tayo et al. (2008) reported the presence of aflatoxin and other metabolites in smoked fish due to *A. flavus* in smoked fish sold in Uyo, Akwa Ibom State, Nigeria and confirmed that consumers could have been at risk of aflatoxin poison.

According to Aberoumand (2010), *Escherichia coli* is a classic example of enteric bacteria causing gastroenteritis. *E. coli* including other coliforms and bacteria such as *Staphylococcus* spp. and sometimes enterococci are commonly used as indices of hazardous conditions during processing of fish. Scientists have shown that the contamination of food of fish origin with pathogenic *E. coli* probably occur during handling of fish and during the production process (Jimoh et al., 2009). The micro-organisms associated with smoked fish pose a great threat to the populace as the transfer of the micro-organisms attack the immune system of the consumer, usually man, thereby, giving room for the invasion of disease. *E. coli* and *Staphylococcus aureus* were reported as the predominant microorganisms present in smoked fish in Asaba area of Delta State of Nigeria (Okonta and Ekelemu, 2005). Outbreak of Listerosis in different parts of the world in the last three decades as a result of eating smoked fish has been a major public health concern.

This study is therefore, embarked on to assess the microbiological quality of traditional smoked catfish (*Chrysichthys nigrodigitatus*) and by so doing, identify bacterial and fungal species prevalent in traditional smoked catfish, their distribution, effects and possible public health implications of the presence of such microorganisms.

METHODOLOGY

Fish used

Fresh fish samples were obtained from ten different fishing communities of Badagry and Epe Local Government Areas of Lagos State, Nigeria. The fresh silver catfish samples were freshly harvested. The samples were taken to the IFSERAR laboratory, Federal University of Agriculture, Abeokuta for smoking.

Area of study

Using a current geopolitical map of Nigeria (Figure 1), Lagos State lies to the south-western part of Nigeria with 20 local government areas (LGAs) and has boundaries with Ogun State both in the north and east. It is bordered on the west by the Republic of Benin and in the south, stretches for 180 km along the coast of the Atlantic Ocean. It therefore has 22.5% of Nigeria's coastline and occupies an area of 3,577 sq km land mass with about 786.94 sq km (22%) of it being lagoons and creeks. The state is endowed with marine, brackish and fresh water ecological zones with varying fish species that provide productive fishing opportunity for fishermen. Two local government areas (Badagry and Epe Local Government) were covered because they are highly densed fish processing centers. They were selected for the study and hazard analyses of the products.

Sampling procedure

Fresh silver catfish (100 samples) and smoked silver catfish (100 samples) were collected from 10 processing centres from each of the two local government areas by purposive sampling in sterile containers (Ziploc).

All freshly harvested silver catfish samples were kept on ice during transportation to the laboratory and smoked on the same day. Smoked fish samples were analyzed immediately.

Fish smoking process

Smoked fish was prepared following the method as described by Crapo (2011) with modifications. Fish were carefully cleaned to remove slime, blood and harmful bacteria. The fish were eviscerated, leaving the skin on the fish. The fish were cut into uniform pieces (fillet) so that no parts will get overheated.

The fish were smoked to 80°C internal temperature (with a thermometer) for at least 24 h. The kiln temperature was adjusted as needed throughout this smoking period to maintain the 80°C internal temperature. Hands, utensils and work surfaces were cleaned when transferring fish from smoker to oven to cool down to avoid cross-contamination. Smoking was done for 24 h until the fish was fully dried.

Physico-chemical analysis

Kent pH meter (Kent Ind. Measurement Ltd., survey) model 7020 equipped with a glass electrode was used to measure the pH of the flesh, employing 10 g of fish homogenized in 10 ml of distilled water. Triplicate determinations were made in all cases. The pH

Figure 1. Map of Lagos State showing the 20 LGA (nigerianmuse.com accessed December 3, 2014).

meter was calibrated using pH 4.0 and 7.0 buffers. All chemicals used in this study were of the analytical grade unless stated otherwise.

Microbiological studies

The presence of pathogens in fresh and smoked fish samples was investigated. These include: *Listeria monocytogenes, Salmonella* Paratyphi, *E. coli*, *S. aureus* and fungal count. Fish samples (fresh and smoked) obtained from the identified processing centres were analyzed microbiologically. The microbiological procedures recommended in the International Commission on Microbiological Specification for Foods (ICMSF, 1986) were applied. Culture media were those of Oxoid, Biolife and Difco. For each sample, 25 g were weighed out and transferred to a sterile blender with 225 ml of 0.1% peptone and mixed thoroughly for 2 min to prepare fish homogenate. These were then analyzed as follows.

Total viable bacterial counts

Appropriate dilutions of the fish homogenate were prepared and inoculated onto sterile Petri dishes. Plate count agar (Oxoid) media were then poured. Plates were incubated at 35–37°C for 48 h and colonies were then counted and reported as total colony count/ml. A second set of plates was incubated at 35–37°C for 48 h in a carbon dioxide incubator or under anaerobic conditions using a gas pack anaerobic jar. Colonies were then counted and reported as anaerobic total bacterial count. In case of spore formers count, the food homogenate was boiled first at 75–80°C and then rapidly cooled. Appropriate serial dilutions were prepared and inoculated onto the surface of sterile and dried plate count agar media. These were incubated finally at 35–37°C for 48 h.

Detection of *Escherichia coli*

1 ml of each of the decimal dilutions of the fresh and smoked fish homogenate was plated on poured Eosine Methylene Blue Agar (Oxoid) and then incubated at 35–37°C for 24 h. Counts were calculated from the number of growth on the plates. The colonies with green metallic sheen were counted as *E. coli*.

Detection of *S. aureus*

A sample of 0.1 ml of the fresh and smoked fish homogenate and dilutions was inoculated on Baird-Parker (Difco) agar plates and incubated at 35–37°C for 48 h. Colonies appearing to be black and shiny with narrow white margins and surrounded by clear zones were identified by coagulase test reactions. The coagulase test was carried out by first inoculating typical colonies in brain heart infusion broth (Difco) and incubating at 37°C for 24 h. From the resulting cultures, 0.1 ml was then added to 0.3 ml of rabbit plasma in sterile tubes and incubated at 37°C for 4 h. The formation of a distinct clot was evidence of coagulase activity.

Detection of *Salmonella* Paratyphi

Samples of fresh and smoked fish homogenate and dilutions were inoculated in Salmonella-shigella agar (Oxoid) and incubated at 35–37°C for 24 h. For identification, 2–3 suspected colonies were inoculated into tryptone broth for indole test, triple sugar iron agar slant (Oxoid), urea broth and lysine iron agar. These were incubated at 37°C for 24 h. *Salmonella* species is indole negative, on triple sugar iron it produces acid (yellow) and alkaline (red) with or without gas and hydrogen sulfide, is urea negative, and on lysine iron agar shows an alkaline (purple) reaction throughout the medium. Serological tests were then carried out.

Detection of *L. monocytogenes*

A sample of 0.1 ml of the fresh and smoked fish homogenate and dilutions was inoculated on Brilliant Listeria Agar (Oxoid) plates and incubated at 35–37°C for 24 h. Colonies appearing were counted and reported as *L. monocytogenes*.

Table 1. Microbial quality (cfu/g) and pH of fresh silver catfish (*Chrysichthys nigrodigitatus*) from 20 different processing centres.

Locations	*Listeria monocytogenes*	*Salmonella* Paratyphi	*E.coli*	Staphylococcal count	Fungal count	T.V.C.	pH
Agbalata	$1.9 \times 10^{2}_{a}$	$1.0 \times 10^{2}_{a}$	$1.3 \times 10^{2}_{ab}$	$5.4 \times 10^{2}_{ab}$	-	$7.8 \times 10^{8}_{cd}$	6.96ab
Ajido	$1.8 \times 10^{2}_{a}$	$1.3 \times 10^{2}_{ab}$	$1.1 \times 10^{2}_{a}$	$5.1 \times 10^{2}_{a}$	-	$6.9 \times 10^{8}_{ab}$	7.04_{bcd}
Asakpo	$2.3 \times 10^{2}_{abc}$	$1.1 \times 10^{2}_{a}$	$1.2 \times 10^{2}_{ab}$	$6.4 \times 10^{2}_{cd}$	-	$7.2 \times 10^{8}_{ab}$	6.91ab
Boguru	$2.0 \times 10^{2}_{ab}$	$1.4 \times 10^{2}_{bc}$	$1.5 \times 10^{2}_{bc}$	$6.1 \times 10^{2}_{bcd}$	-	$8.0 \times 10^{8}_{de}$	6.98ab
Fvanoveh	$2.4 \times 10^{2}_{abc}$	$1.0 \times 10^{2}_{a}$	$1.2 \times 10^{2}_{ab}$	$6.3 \times 10^{2}_{cd}$		$6.5 \times 10^{8}_{a}$	7.01abc
Gberefun	$2.6 \times 10^{2}_{bcd}$	$1.2 \times 10^{2}_{ab}$	$1.4 \times 10^{2}_{bc}$	$5.8 \times 10^{2}_{bc}$	-	$7.6 \times 10^{8}_{cd}$	7.13cdef
Gbetrome	$2.1 \times 10^{2}_{ab}$	$1.1 \times 10^{2}_{a}$	$1.2 \times 10^{2}_{ab}$	$5.6 \times 10^{2}_{ab}$	-	$7.3 \times 10^{8}_{bc}$	6.99ab
Ilaje	$2.5 \times 10^{2}_{bcd}$	$1.0 \times 10^{2}_{a}$	$1.0 \times 10^{2}_{a}$	$5.8 \times 10^{2}_{bc}$	-	$6.8 \times 10^{8}_{a}$	7.15def
Kofegameh	$2.3 \times 10^{2}_{abc}$	$1.2 \times 10^{2}_{ab}$	$1.2 \times 10^{2}_{ab}$	$6.1 \times 10^{2}_{bcd}$	-	$8.3 \times 10^{8}_{ef}$	6.90a
Pako	$2.0 \times 10^{2}_{ab}$	$1.4 \times 10^{2}_{bc}$	$1.0 \times 10^{2}_{a}$	$6.0 \times 10^{2}_{bc}$	-	$8.0 \times 10^{8}_{de}$	7.23f
Afuye	$2.3 \times 10^{2}_{abc}$	$1.0 \times 10^{2}_{a}$	$1.3 \times 10^{2}_{ab}$	$6.3 \times 10^{2}_{cd}$	-	$6.6 \times 10^{8}_{a}$	7.18ef
BodinYawa	$2.1 \times 10^{2}_{ab}$	$1.2 \times 10^{2}_{ab}$	$1.0 \times 10^{2}_{a}$	$5.2 \times 10^{2}_{a}$	-	$7.4 \times 10^{8}_{bc}$	6.93ab
Idale	$2.4 \times 10^{2}_{abc}$	$1.4 \times 10^{2}_{bc}$	$1.3 \times 10^{2}_{ab}$	$5.3 \times 10^{2}_{a}$	-	$8.8 \times 10^{8}_{g}$	6.95ab
Igbodun	$2.1 \times 10^{2}_{ab}$	$1.0 \times 10^{2}_{a}$	$1.1 \times 10^{2}_{a}$	$6.4 \times 10^{2}_{cd}$	-	$7.5 \times 10^{8}_{bcd}$	7.24f
Ilogun	$2.3 \times 10^{2}_{abc}$	$1.2 \times 10^{2}_{ab}$	$1.4 \times 10^{2}_{bc}$	$6.8 \times 10^{2}_{de}$	-	$6.6 \times 10^{8}_{a}$	7.16def
Mejona	$2.5 \times 10^{2}_{a}$	$1.5 \times 10^{2}_{bc}$	$1.0 \times 10^{2}_{a}$	$5.0 \times 10^{2}_{a}$	-	$8.4 \times 10^{8}_{ef}$	6.94ab
Oluwo	$2.1 \times 10^{2}_{ab}$	$1.3 \times 10^{2}_{ab}$	$1.1 \times 10^{2}_{a}$	$5.6 \times 10^{2}_{ab}$	-	$8.0 \times 10^{8}_{de}$	7.12cdef
Okorisan	$2.0 \times 10^{2}_{ab}$	$1.0 \times 10^{2}_{a}$	$1.3 \times 10^{2}_{ab}$	$6.3 \times 10^{2}_{cd}$	-	$8.1 \times 10^{8}_{de}$	7.05bcde
Orita	$2.5 \times 10^{2}_{bcd}$	$1.2 \times 10^{2}_{ab}$	$1.0 \times 10^{2}_{a}$	$6.1 \times 10^{2}_{bcd}$	-	$7.4 \times 10^{8}_{bc}$	7.14def
Orogoro	$2.3 \times 10^{2}_{abc}$	$1.4 \times 10^{2}_{ab}$	$1.2 \times 10^{2}_{ab}$	$5.4 \times 10^{2}_{ab}$	-	$8.3 \times 10^{8}_{ef}$	6.99ab

Data are means of 3 replicates. Data with different subscripts in the same column indicate significant difference at $p<0.05$. T.V.C = total viable count; - = no count.

Enumeration of fungi

Appropriate dilutions of Sabouraud dextrose agar plates (Oxoid) were poured over 1 ml of the fish homogenate and dilutions. Plates were incubated at 22–25°C for 3 days and then colonies were counted and reported as fungal count/ml.

Data analysis

All data analyses were done in triplicates. The data obtained were subjected to descriptive statistics using IBM SPSS version 21.0 software. One way analysis of variance (ANOVA) was done using Duncan's multiple range test ($p<0.05$) to study the difference between means.

RESULTS AND DISCUSION

pH is the most critical factors affecting microbial growth and spoilage of foods. The pH value of fresh silver catfish samples (Table 1), ranged from 6.90 – 7.24 and pH value of smoked silver catfish samples ranged from 6.27 – 6.86 and 6.5 – 6.86. The pH values of the fresh fish samples was high, this may be due to biochemical reactions and enzyme action as a result of delay in reaching the shore from the sea because most of the fishermen had no cooling system in their boats or canoes. However, the pH in fish tissues drops due to smoking (Doe, 1998; da Silva, 2002; da Silva et al., 2008). A study on the absence and presence of pathogens such as *Listeria, Salmonella, Staphylococcus,* and *Escherichia coli* was conducted to evaluate microbial safety and quality of smoked silver catfish. The result indicates the predominance of *L. monocytogenes, S. aureus*, *S.* Paratyphi and *E. coli* in the fresh and smoked silver catfish samples. The results of the microbiological study (Table 1) indicated that total viable count (TVC) of fresh fish samples increased significantly ($p<0.05$). Total plate count (TVC) of fresh silver catfish samples was $6.6 \times 10^{6} – 8.8 \times 10^{8}$ cfu/g and TVC of samples of smoked silver catfish (Table 2) were 2.0×10^{4} - 8.6×10^{4} cfu/g and $1.0 \times 10^{3} – 5.4 \times 10^{3}$ cfu/g (Table 2). The TVC values obtained for the smoked silver catfish samples were within the range of specified microbiological limits recommended by ICMSF (1986) for fish and fishery products, the maximum recommended bacterial counts for good quality products (m) is 5×10^{5} (5.7 log10 CFU/g). *L. monocytogenes* count of fresh silver catfish samples (Table 1) was 1.8×10^{2} - 2.5×10^{2} cfu/g and that of samples of smoked silver catfish from different processing centres (local drum kiln) ranged from $1.3 \times 10^{1} – 13.2 \times 10^{1}$ cfu/g (Table 2). Although the *L. monocytogenes* count values obtained for the smoked silver catfish samples were low, the range of specified microbiological limits recommended by ICMSF (1986)

Table 2. Microbial quality (cfu/g) and pH of smoked silver catfish (*Chrysichthys nigrodigitatus*) from 20 different processing centres using local drum kiln and conventional smoke kiln.

Locations	*Listeria Monocytogenes*		*Salmonella* Paratyphi		*E. coli*		Staphylococcal count		Fungal count		T.V.C.		pH	
	Local	Convent	Local	Convent	Local	Convent	Local	Convent	Local	Convent	Local	Convent	Local	Convent
Agbalata	$4.0 \times 10^{1}_{c}$	-	-	-	-	-	$23.4 \times 10^{2}_{g}$	$2.5 \times 10^{1}_{cd}$	-	-	$4.6 \times 10^{4}_{c}$	$4.4 \times 10^{3}_{f}$	6.43_{abcde}	6.51_{a}
Ajido	$1.4 \times 10^{1}_{a}$	-	-	-	-	-	$17.1 \times 10^{2}_{d}$	$3.5 \times 10^{1}_{ef}$	-	-	$6.1 \times 10^{4}_{f}$	$5.3 \times 10^{3}_{g}$	6.48_{bcdef}	6.73_{ef}
Asakpo	$6.1 \times 10^{1}_{d}$	-	-	-	-	-	$39.0 \times 10^{2}_{k}$	-	-	-	$5.4 \times 10^{4}_{de}$	$1.0 \times 10^{3}_{a}$	6.67_{gh}	6.62_{bcd}
Boguru	$7.5 \times 10^{1}_{ef}$	-	-	-	-	-	$60.2 \times 10^{2}_{n}$	-	-	-	$5.0 \times 10^{4}_{d}$	$1.1 \times 10^{3}_{a}$	6.62_{fgh}	6.66_{cdef}
Fvanoveh	$11.0 \times 10^{1}_{g}$						$30.5 \times 10^{2}_{i}$	$1.4 \times 10^{1}_{ab}$			$2.5 \times 10^{4}_{a}$	$1.8 \times 10^{3}_{cd}$	6.54_{cdefg}	6.61_{def}
Gberefun	$5.4 \times 10^{1}_{cd}$	-	-	-	-	-	$14.3 \times 10^{2}_{c}$	-	-	-	$4.3 \times 10^{4}_{c}$	$1.5 \times 10^{3}_{bc}$	6.41_{abcd}	6.90_{g}
Gbetrome	$8.2 \times 10^{1}_{f}$	-	-	-	-	-	$18.6 \times 10^{2}_{e}$	$2.4 \times 10^{1}_{cd}$	-	-	$2.6 \times 10^{4}_{a}$	$1.0 \times 10^{3}_{a}$	6.73_{h}	6.68_{def}
Ilaje	16.0×10^{1}	-	-	-	-	-	$25.0 \times 10^{2}_{h}$	$3.2 \times 10^{1}_{e}$	-	-	$2.0 \times 10^{4}_{a}$	$1.2 \times 10^{3}_{a}$	6.39_{abc}	6.73_{ef}
Kofegameh	$12.3 \times 10^{1}_{h}$	-	-	-	-	-	$34.2 \times 10^{2}_{i}$	-	-	-	$5.5 \times 10^{4}_{de}$	$1.3 \times 10^{3}_{ab}$	6.46_{bcdef}	6.86_{g}
Pako	$2.6 \times 10^{1}_{b}$	-	-	-	-	-	$19.5 \times 10^{2}_{f}$	$3.1 \times 10^{1}_{e}$	-	-	$3.3 \times 10^{4}_{b}$	$1.1 \times 10^{3}_{a}$	6.55_{cdefg}	6.75_{f}
Afuye	$2.5 \times 10^{1}_{b}$	-	-	-	-	-	$5.6 \times 10^{2}_{a}$	-	-	-	$2.4 \times 10^{4}_{a}$	$1.0 \times 10^{3}_{a}$	6.42_{abcde}	6.57_{abc}
BodinYawa	$4.1 \times 10^{1}_{c}$	-	-	-	-	-	$18.0 \times 10^{2}_{e}$	$3.2 \times 10^{1}_{e}$	-	-	$4.1 \times 10^{4}_{c}$	$3.5 \times 10^{3}_{e}$	6.57_{cdefgh}	6.69_{cde}
Idale	$1.3 \times 10^{1}_{a}$	-	-	-	-	-	$18.1 \times 10^{2}_{e}$	$1.2 \times 10^{1}_{a}$	-	-	$4.5 \times 10^{4}_{c}$	$1.4 \times 10^{3}_{ab}$	6.43_{abcde}	6.52_{a}
Igbodun	$5.1 \times 10^{1}_{cd}$	-	-	-	-	-	$56.5 \times 10^{2}_{m}$	-	$8.0 \times 10^{1}_{c}$	-	$6.3 \times 10^{4}_{f}$	$1.2 \times 10^{3}_{a}$	6.41_{abcd}	6.63_{cde}
Ilogun	$7.0 \times 10^{1}_{e}$	-	-	-	-	-	$39.2 \times 10^{2}_{k}$	$1.3 \times 10^{1}_{ab}$	$8.2 \times 10^{1}_{c}$	-	$3.1 \times 10^{4}_{b}$	$1.3 \times 10^{3}_{ab}$	6.54_{cdefg}	6.74_{f}
Mejona	$8.5 \times 10^{1}_{f}$	-	-	-	-	-	$45.0 \times 10^{2}_{l}$	-	$4.1 \times 10^{1}_{a}$	-	$3.3 \times 10^{4}_{b}$	5.4×10^{3}	6.60_{efgh}	6.65_{cdef}
Oluwo	$6.0 \times 10^{1}_{d}$	-	-	-	-	-	$60.4 \times 10^{2}_{n}$	-	$8.0 \times 10^{1}_{c}$	-	$5.0 \times 10^{4}_{d}$	$1.1 \times 10^{3}_{a}$	6.63_{fgh}	6.53_{ab}
Okorisan	$13.2 \times 10^{1}_{i}$	-	-	-	-	-	-	$2.1 \times 10^{1}_{c}$	-	-	$2.2 \times 10^{4}_{a}$	$1.4 \times 10^{3}_{ab}$	6.58_{defgh}	6.84_{g}
Orita	$16.0 \times 10^{1}_{i}$	-	-	-	-	-	$30.2 \times 10^{2}_{i}$	-	-	-	$4.5 \times 10^{4}_{c}$	$2.0 \times 10^{3}_{cd}$	6.27_{a}	6.59_{abcd}
Orogoro	$11.4 \times 10^{1}_{g}$	-	-	-	-	-	$8.5 \times 10^{2}_{b}$	$1.0 \times 10^{1}_{a}$	$7.0 \times 10^{1}_{b}$	-	$5.1 \times 10^{4}_{d}$	$1.4 \times 10^{3}_{ab}$	6.32_{ab}	6.73_{ef}

Data are means of 3 replicates. Data with different subscripts in the same column indicate significant difference at $p<0.05$. T.V.C = total viable count; - = no count.

for *L. monocytogenes* for fish and fishery products is the presence of the organism, that is zero tolerance so most of the samples from local drum kiln do not meet the ICMSF recommended microbial specification. Therefore, the smoked silver catfish samples from all processing centres need to be cooked before consumption in order to destroy *L. monocytogenes* that is present in the smoked silver catfish to prevent possibility of food poison by listeriosis. All the smoked silver catfish samples of convention smoke kiln tested negative for *L. monocytogenes* while the fresh fish samples contained *L. monocytogenes*. Goktepe and Moody (1998) reported that *Listeria* spp. counts of raw catfish fillets were 4.37 log CFU/g; after brining, the count decreased slightly to 3.24 log CFU/g and no *Listeria* spp. was detected in samples after hot smoking. *Staphylococcal* count of fresh silver catfish samples (Table 1) ranged from 5.0×10^{3} - 6.4×10^{3} cfu/g and that of samples of smoked silver catfish from different processing centres (local drum kiln) ranged from 5.6×10^{2} - 60.4×10^{2} cfu/g and 1.0×10^{2} – 3.5×10^{2} cfu/g (Table 2). The *Staphylococcal* count values obtained for the smoked silver catfish were below the specified recommended value for all fish. The *S. aureus* safety level is equal to or greater than 10^{4}/g and in many cases, these levels represent the point at or above which the agency will take legal action to remove products from the market (FDA, 2001). In addition, smoking also reduced *Staphylococci* and fungal counts. The isolation of *Staphylococcus* in smoked samples can be attributed to post processing contamination. *S.* Paratyphi was not detected in smoked silver catfish samples obtained using local drum kiln and

conventional smoke kiln and this conformed with the specified microbiological limits recommended by ICMSF (1986) for *S. paratyphi* count for fish and fishery products which is the presence of the organism, that is, zero tolerance. In all cases, this suggests Good Manufacturing Practices (GMP) and no faecal contamination of the products as *Salmonella paratyphi* and *E. coli* serve as indicator organisms for faecal contamination of foods. In this study, fungal count of samples of smoked silver catfish from different processing centres (local drum kiln) ranged from 1.1×10^1 - 10.0×10^1 cfu/g (Table 2). The populations of fungi in the samples were all below $5x10^5$ CFU/g specified microbiological limits recommended by ICMSF (1986) for fungi, except for the samples from convention smoke kiln that had no fungi count.

Conclusion

The pH values of the fresh fish samples were found to be high. However, the pH in fish tissues drops due to smoking. From this study, smoking significantly ($p<0.05$) reduced the pH and total viable count in all samples of smoked silver catfish using local drum kiln; however, the samples of smoked fish using conventional smoke kiln showed no count for *L. monocytogenes. S. paratyphi and E. coli* were not detected in all smoked silver catfish samples obtained using local drum kiln and conventional smoke kiln and this conformed with the specified microbiological limits recommended by ICMSF (1986) for *S. paratyphi* and *E. coli* count for fish and fishery products which is the presence of the organisms, that is, zero tolerance. In all cases, this suggests GMP and no faecal contamination of the products as *S. paratyphi and E. coli* serve as indicator organisms for faecal contamination of foods.

Conflict of interests

The authors did not declare any conflict of interest.

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Inducing salinity tolerance in chickpea (*Cicer arietinum* L.) by inoculation of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase-containing *Mesorhizobium* strains

Deepika Chaudhary and Satyavir Singh Sindhu*

Department of Microbiology, CCS Haryana Agricultural University, Hisar- 125004, India.

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Salinity stress severely affects growth, nodulation and yield of chickpea (*Cicer arietinum* L.). However, inoculation with *Mesorhizobium* strains containing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase improves the plant growth by reducing the level of ethylene induced by salt stress. Fifty (50) *Mesorhizobium* isolates were obtained from nodules of chickpea plants on yeast extract mannitol agar (YEMA) medium. *Mesorhizobium* isolates were screened for ACC utilization and growth at different salt concentrations in YEMA medium. Six salt tolerant *Mesorhizobium* isolates were checked for their role in plant growth promotion under pot house conditions in chillum jar assembly. *Mesorhizobium* strains having ACC utilization ability caused an increase in the nodule number, nodule weight and shoot dry weight after plant growth for 50 and 80 days, both with and without NaCl. *Mesorhizobium* isolate MBD26 showed 294 mg/plant shoot dry weight without salt condition after 50 days of plant growth. *Mesorhizobium* isolate MBD26 increased shoot dry weight by 49.52% (without salt) and 41.53% in the presence of salt (40 mM NaCl) after 80 days of plant growth. It was observed that inoculation with *Mesorhizobium* isolates containing ACC-deaminase improved nodulation and plant growth of chickpea over ACC deaminase lacking isolates. Thus, inoculation with *Mesorhizobium* strains possessing ACC utilization ability could be a sustainable approach to improve plant growth under salinity stress.

Key words: 1-Aminocyclopropane-1-carboxylic acid (ACC) utilization, salt stress, *Mesorhizobium*, chickpea, nodulation, plant growth.

INTRODUCTION

Maintenance of sustainable agricultural crop productivity and simultaneously increasing food production to meet the demands of growing human population is a challenging task. Moreover, abiotic stresses due to the climate changes, soil environment and agricultural practices adversely affect the crop productivity. The soil environment is constantly changing, making it relatively stressful for both macro- and micro-organisms.

*Corresponding author. E-mail: sindhuss58@gmail.com. Tel: +911662-230875.

Changes such as fluctuations in pH, temperature, salinity and nutrient availability greatly influence the growth, survival and metabolic activity of soil microorganisms (Zahran, 1999). Soil salinity affects about 800 Mha of arable lands worldwide (Munns and Tester, 2008) and this area is gradually expanding. Salinity affects agricultural production in arid and semiarid regions, where rainfall is limited and is not sufficient to transport salts from the plant root zone (Tester and Davenport, 2003).

Salt concentration negatively affects the growth and yield of legume plants by its harmful effect on biological nitrogen fixation, lessening supply of photosynthates to nodule of plants (Bekki et al., 1987), reduced supply of respiratory substrates supply to bacteroids (Delgado et al., 1994) and modifications in the diffusion barrier of oxygen (Serraj et al., 1994). Salt stress also enhance ethylene (C_2H_4) synthesis which in most of the cases acts as stress hormone in plant (Grichko and Glick, 2001; Arshad and Frankenberger, 2002). ACC deaminase enzyme lowers the level of C_2H_4 in plants and it protects the plants from the deleterious effects of environmental stresses (Reed and Glick, 2005; Saleem et al., 2007; Aamir et al., 2013).

Recently, plants inoculated with plant growth promoting rhizobactertia containing ACC deaminase activity have been found to thrive through the salinity menace leading to normal growth pattern (Mayak et al., 2004a; Saravanakumar and Samiyappan, 2006; Gamalero et al., 2010). Mayak et al. (2004b) reported that inoculation with rhizobacterial strains dramatically lowered the level of ethylene and growth inhibition of tomato plants was prevented when grown in the presence of high concentration of salts.

The frequency of ACC deaminase activity containing bacterial strains is relatively low and 12% of isolated *Rhizobium* spp. from various sites in Southern and Central Saskatchewan were found to possess this enzyme (Duan et al., 2009). ACC deaminase activity has been found in a wide range of bacterial isolates including *Azospirillum*, *Rhizobium*, *Agrobacterium*, *Achromobacter*, *Burkholderia*, *Ralstonia*, *Pseudomonas* and *Enterobacter* (Glick et al., 2007a; Saleem et al., 2007; Ahmad et al., 2011; Khandelwal and Sindhu, 2012).

In this study, *Mesorhizobium* isolates were obtained from legume root nodules collected from chickpea plants grown under saline areas. Selected ACC deaminase containing *Mesohizobium* isolates were evaluated for growth promotion of chickpea under salinity stress conditions.

MATERIALS AND METHODS

Isolation of *Mesorhizobium* from chickpea nodules

Healthy chickpea plants (with nodules) grown in saline soils were collected from different locations in Hisar, Bhiwani and Sirsa districts of Haryana state. Nodules were surface sterilized with $HgCl_2$ (1%), crushed with sterile glass rod and the crushed nodule suspension was streaked on yeast extract mannitol agar (YEMA) medium plates (Garg et al., 1985). The plates were incubated for three to eight days at 28±2°C. Purified cultures were maintained at 4°C in the refrigerator till further use.

Screening of *Mesorhizobium* isolates for growth at different salt concentrations

Purified *Mesorhizobium* isolates were checked for their ability to grow at different concentrations of sodium chloride (NaCl), that is, 1, 2, 3 and 4% (w/v), on YEMA medium plates containing 20 mM HEPES (N-2-hydroxyethane-sulphonic acid) (Marsudi et al., 1999). Medium plates were spotted with a loopful of bacterial isolates. The plates were incubated for three to four days at 28±2°C in a biological oxygen demand (B.O.D.) incubator. The susceptibility to NaCl was recorded as a positive or negative result.

ACC utilization by *Mesorhizobium* isolates

Minimal medium plates supplemented with 2 mM ACC were prepared (Penrose and Glick, 2003). A loopful of 48-h old growth of *Mesorhizobium* isolate was spotted on the ACC supplemented medium plates (Khandelwal and Sindhu, 2012). The minimal medium incorporated with ammonium sulphate (2 g/L) was kept as control to compare the growth of bacterial isolates to those with ACC supplemented medium plates. The growth of bacterial isolates was recorded after five days of incubation at 28±2°C. The bacterial cultures showing good growth on ACC supplemented medium plates, that is, having high efficiency of ACC utilization as nitrogen source, were scored as bacteria having ACC deaminase activity.

Effect of inoculation on plant growth of chickpea

Selected ACC utilizing and ACC non-utilizing *Mesorhizobium* isolates were checked for nodulation and plant growth using chickpea (*Cicer arietinum* L.) var. HC-1 in sterilized chillum jar assemblies (Dahiya and Khurana, 1981) containing washed river sand in the upper jar and Sloger's nitrogen-free mineral salt solution (Sloger, 1969) in the lower assembly. Surface sterilized seeds of chickpea were inoculated with 5 ml culture (containing 10^7-10^8 cells/ml of growth suspension) of selected ACC utilizing and ACC non-utilizing *Mesorhizobium* isolates individually. Uninoculated seeds were sown as control. Quarter strength Sloger's nitrogen-free mineral salt solution was used for watering whereas salinity levels of 40 mM NaCl were maintained for salt treatment. The observations for shoot biomass, nodule number, nodule weight and plant nitrogen (Lindner, 1944) were recorded at 50 and 80 days of plant growth.

RESULTS

Salt tolerance of different *Mesorhizobium* isolates

Among the fifty *Mesorhizobium* isolates tested at 1, 2, 3 and 4% NaCl concentrations, only four isolates, that is, MHD2, MHD12, MHD14 and MSD41 showed growth up to 4% NaCl concentration (Table 1 and Figure 1). Four

Table 1. Salt tolerance among various *Mesorhizobium* isolates.

Colony size (mm)	Growth of *Mesorhizobium* isolates at different NaCl concentrations			
	1%	2%	3%	4%
0-5 Group 1	MHD6, MHD9, MHD10, MBD16, MBD18, MBD19, MBD22, MBD24, MBD33, MBD40, MBD41, MBD42, MBD43, MBD47	MHD6, MHD9, MHD10, MHD15, MBD19, MBD22, MBD24, MBD25, MBD26, MBD29, MBD30, MBD32, MBD33, MSD38, MSD40, MSD41, MSD42, MBD47	MHD2, MHD6, MHD9, MHD10, MBD19, MBD22, MBD24, MBD25, MBD26, MBD29, MBD30, MBD31, MSD38, MSD40, MSD41	MHD2, MSD41
5-10 Group 2	MHD14, MHD15, MBD20, MBD25, MBD26, MBD27, MBD28, MBD29, MBD30, MBD31, MBD32, MBD34, MSD38	MHD1, MHD2, MHD14, MBD20, MBD27, MBD28, MBD31	MHD1, MHD12, MHD14, MBD20	MHD12, MHD14
10-15 Group 3	MHD1, MHD2, MHD12	MHD12	-	-
No growth Group 4	MHD3, MHD4, MHD5, MHD7, MHD8, MHD11, MHD13, MBD17, MBD21, MBD23, MSD35, MSD36, MSD37, MSD39, MSD44, MSD45, MSD46, MSD48, MSD49, MSD50	MHD3, MHD4,, MHD5, MHD7, MHD8, MHD11, MHD13, MBD16- MBD18, MBD21, MBD23, MBD34-, MSD37, MSD39, MSD43 - MSD46, MSD48 - MSD50	MHD3 - MHD5, MHD7, MHD8, MHD11, MHD13, MHD15, MBD16 - MBD18, MBD21, MBD23, MBD27, MBD28, MBD32 - MBD34, MSD35 - MSD37, MSD39, MSD42 - MSD50	MHD1, MHD3 - MHD11, MHD13, MHD15, MBD16 - MBD34, MSD35 - MSD40, MSD42 - MSD50

major groups were distinguished on the basis of different colony size on salt incorporated medium plates. In the third group, only three *Mesorhizobium* isolates MHD1, MHD2 and MHD12 showed large colony size (10-15 mm) at 1 and 2% NaCl salt concentration. At 2% NaCl concentration, twenty four *Mesorhizobium* isolates did not show growth and only MHD12 isolate showed large colony size. Seven *Mesorhizobium* isolates showed 5-10 mm colony diameter on 2% NaCl plates whereas eighteen isolates of *Mesorhizobium* showed small colony size (group 1st). At 3% salt concentration, fifteen *Mesorhizobium* isolates having small colony growth were found in 1st group, whereas, MHD1, MHD12, MHD14 and MBD20 isolates showed 5-10 mm colony size (group 2). Remaining 62% isolates did not show growth. At 4% NaCl concentration, forty six *Mesorhizobium* isolates did not show growth. Only two isolates MHD12 and MHD14 showed more growth (5-10 mm) than other two isolates MHD2 and MSD41 (Table 1). At 1% salt concentration, twenty *Mesorhizobium* isolates did not show growth (4th group).

ACC utilization by different *Mesorhizobium* isolates

All the *Mesorhizobium* isolates were screened for ACC utilization and *Mesorhizobium* isolates were divided into four major categories based upon ACC utilization (Table 2). Only two *Mesorhizobium* isolates, that is, MHD1 and MHD12 showed significant growth on ACC supplemented plates (Table 2 and Figure 2). Eight isolates, that is, MHD2, MHD4, MHD8, MHD11, MBD25, MBD26, MSD28 and MSD29 moderate growth whereas twelve isolates showed little growth on ACC plates. Twenty eight *Mesorhizobium* isolates did not grow on ACC supplemented plates. On ammonium sulphate containing plates, nine *Mesorhizobium* isolates showed significant growth whereas 33 cultures did not grow. Five *Mesorhizobium* isolates, that is, MBD27, MBD30, MBD33, MSD48 and MSD50 showed little growth.

Symbiotic effectiveness of different *Mesorhizobium* isolates

Three ACC^+ and three ACC^- *Mesorhizobium* isolates were selected for pot house experiment to check their nodulation efficiency and symbiotic effectiveness. Inoculation of *Mesorhizobium* isolate MBD26 showed significant increase in nodule number (54 nodules/plant), nodule weight (357 mg/plant) and nitrogen content (13.67 mg/plant) along with increase in shoot dry weight (294 mg/plant) as compared to uninoculated control (without salt condition) followed by *Mesorhizobium* isolates KR48 and MHD2 after 50 days of plant growth (Table 3). In salt conditions, *Mesorhizobium* isolate MBD26 formed 38

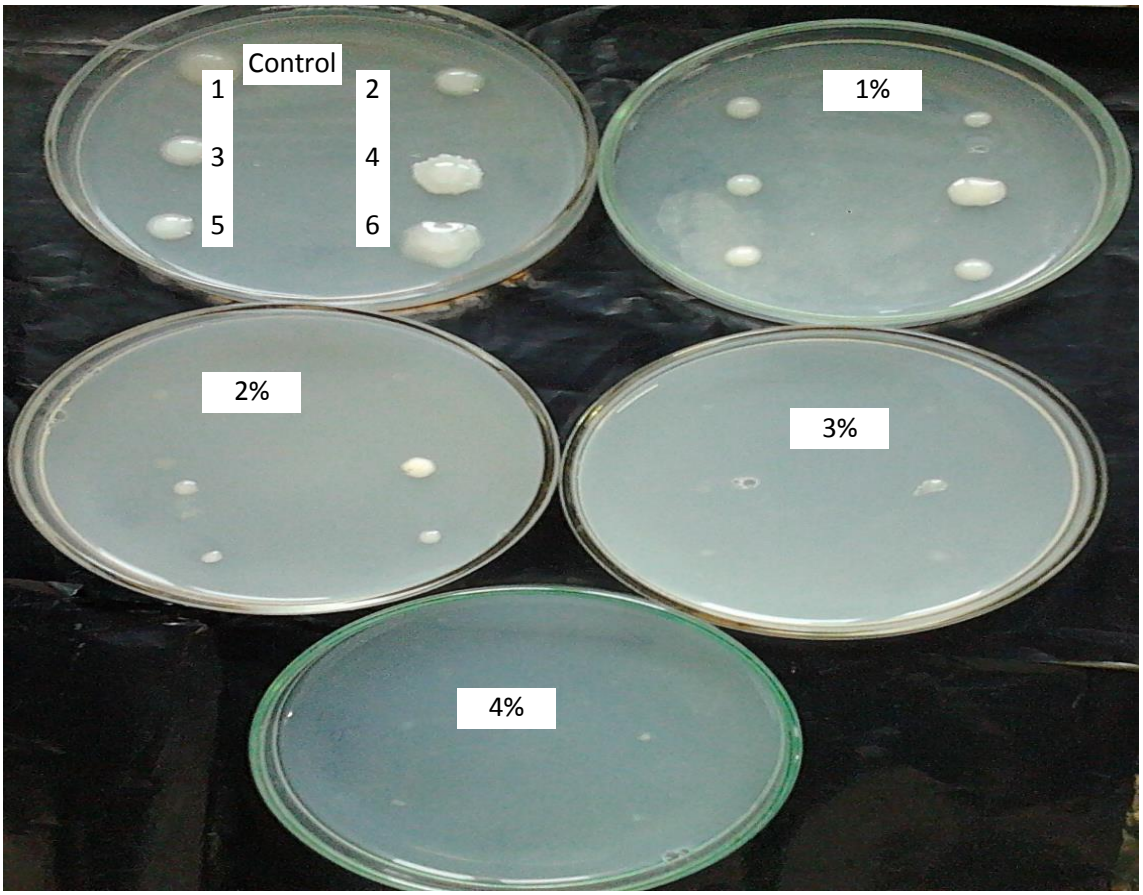

Figure 1. Growth of *Mesorhizobium* isolates on different NaCl concentrations (1-4%) incorporated in YEMA medium. The number given in the figure represents: left to right: 1st row: MBD31, MBD16; 2nd row: MBD28, MBD26; 3rd row: MHD12; MHD2

Table 2. Growth of *Mesorhizobium* isolates on minimal medium supplemented with ACC or ammonium sulphate.

No. of *Mesorhizobium* isolates	Group of ACC utilizing isolates
Growth on ACC supplemented plates	
MHD10. MHD14, MBD27. MBD30 - MBD32, MBD34, MSD40, MSD42, MSD47, MSD48, MSD50	+
MHD2, MHD4, MHD8, MHD11, MBD25, MBD26, MSD28, MSD29	++
MHD1, MHD12	+++
MHD3, MHD5 - MHD7, MHD9, MHD13, MHD15, MBD16 - MBD21, MBD22, MBD23, MBD24, MBD33, MSD35 - MSD39, MSD41, MSD43 - MSD45, MSD46, MSD49	-
Growth on $(NH_4)_2SO_4$ containing plates	
MBD27, MBD30, MBD33, MSD48, MSD50	+
MHD8, MBD32, MSD44	++
MHD1, MHD2, MHD4, MHD11,MHD12, MHD14, MBD25, MBD26, MBD28	+++
MHD3, MHD5 - MHD7, MHD9, MHD10, MHD13, MHD15, MBD16 - MBD24, MBD29, MBD31, MBD34, MSD35 - MSD43, MSD45 - MSD47, MSD49	-

Growth characteristics

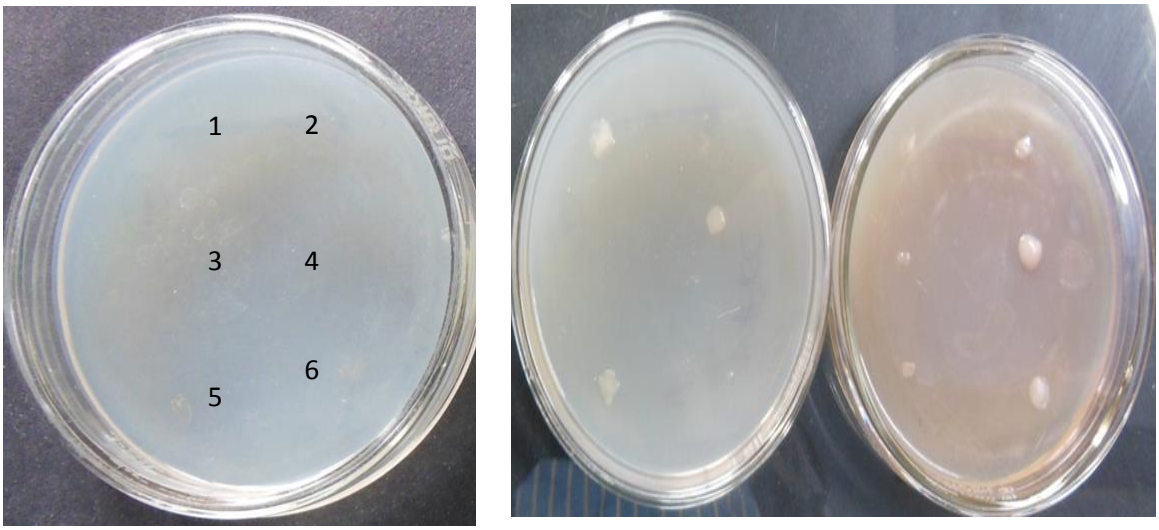

Figure 2. Growth of *Mesorhizobium* isolates on minimal medium (A), ACC supplemented medium (B) and ammonium sulphate amended medium (C). The numbers given in the figure represents: 1, MHD12; 2, MBD33; 3, MSD44; 4, MBD26; 5, MHD2; 6, MHD46

Table 3. Inoculation effect of *Mesorhizobium* isolates in chickpea for nodulation and plant growth under chillum jar conditions at 50 days of plant growth

Inoculation with *Mesorhizobium* isolates	No. of nodules /plant	Nodule weight (g/plant)	Shoot dry weight (g/plant)	Total nitrogen (mg)
Control	-	-	208	8.80
	-	-	140	6.30
MHD9	35	287	230	9.33
	23	131	146	6.57
MBD20	41	298	248	10.67
	25	142	157	7.06
MSD46	43	303	260	11.67
	28	153	162	7.29
MBD26	54	357	294	13.67
	38	192	189	8.50
MHD2	46	317	263	11.50
	29	157	173	7.78
KR48	49	337	281	13.0
	34	173	186	8.37

Values in the second line of each treatment represent the values observed when plants were grown under salt conditions.

nodules/plant having nodule weight (192 mg/plant), shoot dry weight (189 mg/plant) and nitrogen content (8.5 mg/plant) whereas *Mesorhizobium* isolate KR48 formed 34 nodules/plant and produced 186 mg/plant shoot dry weight.

At 80 days of plant growth (without salt condition), inoculation with *Mesorhizobium* isolate MBD26 resulted in nodule weight of 372 g/plant and 471 g/plant shoot dry weight was observed. Maximum plant growth stimulation and nodule formation was observed by inoculation of *Mesorhizobium* isolate MBD26 followed by KR48 and MHD2. It was observed that nodule number, nodule weight, shoot biomass and nitrogen content were increased in *Mesorhizobium* inoculated plants, as compared to uninoculated control plants (Table 4 and Figure 3). The maximum increase in shoot dry weight (49.52%) was recorded by inoculation with MBD26 without salt conditions and 41.53% increase was observed

Table 4. Inoculation effect of *Mesorhizobium* isolates in chickpea on nodulation and plant growth under chillum jar conditions at 80 days of plant growth.

Inoculation with *Mesorhizobium* isolates	No. of nodules /plant	Nodule weight (mg/plant)	Shoot dry weight (mg/plant)	Total nitrogen (mg)
Control	-	-	315	10.39
	-	-	195	7.60
MHD9	47	325	365	12.10
	28	147	215	8.38
MBD20	52	334	397	13.18
	31	153	232	9.04
MSD46	55	337	421	13.97
	33	156	243	9.47
MBD26	65	372	471	15.97
	42	178	276	10.76
MHD2	59	348	434	14.71
	35	167	257	10.02
KR48	62	353	456	15.46
	38	174	269	10.49

Values in the second line of each treatment represent the values observed when plants were grown under salt conditions.

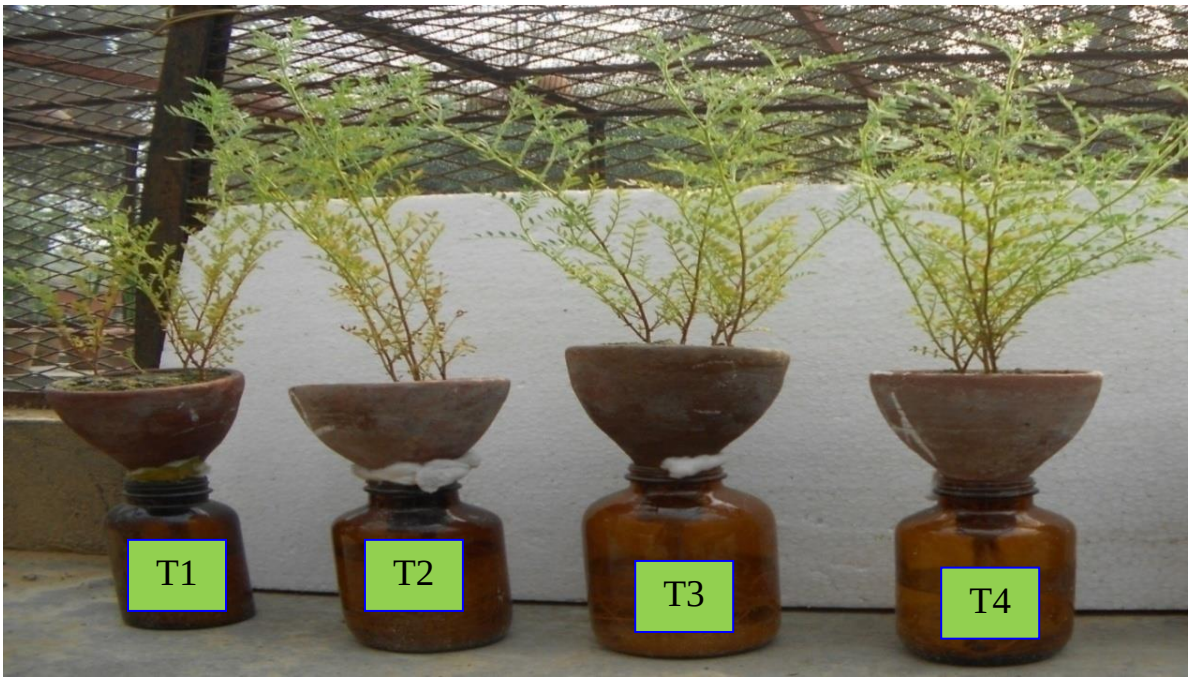

Figure 3. Inoculation effect of *Mesorhizobium* isolates on nodulation and plant growth of chickpea after 80 days under chillum jar conditions. T1: Uninoculated control; T2: *Mesorhizobium* KR48, ACC^+; T3: *Mesorhizobium* MHD2, ACC^+; T4: *Mesorhizobium* MBD26, ACC^+.

in the presence of salt (40 mM NaCl) after 80 days of plant growth. Inoculation with other ACC^+ *Mesorhizobium* isolates also showed similar enhancement for nodule number, nodule weight and nitrogen content as compared to uninoculated control plants. While ACC^- *Mesorhizobium* isolates showed comparatively less

increase in plant parameters as compared to control uninoculated plants. Maximum increase in shoot dry weight (by 24.61%) was observed by inoculation of ACC^- *Mesorhizobium* isolate MSD46 after 80 days in salt treatment. It formed 33 nodule/plant and showed 9.47 mg nitrogen content.

DISCUSSION

Biological nitrogen fixation is performed by a limited group of prokaryotic microorganisms known as diazotrophic bacteria and the nitrogenase enzyme complex of these organisms convert atmospheric inert nitrogen to plant utilizable ammonia form (Araujo et al., 2014). It is known that these microorganisms produce various plant growth-promoting substances (Sindhu et al., 2010; Malik and Sindhu, 2011). Such diazotrophic bacteria have the potential to be used as biofertilizers in different crops for a sustainable agriculture.

Fifty isolates of *Mesorhizobium* were obtained from the nodules of chickpea plants grown in salt affected fields by streaking crushed nodule suspension on the YEMA medium plates. Ogutcu et al. (2010) evaluated the symbiotic effectiveness of *Rhizobium leguminosarum* bv. *Ciceri* strains isolated from perennial wild chickpeas (*Cicer anatolicum*) in comparison with uninoculated control under NaCl salinity stress conditions. Dry weights of root and shoot, root-to-shoot ratio (RSR), number and dry weights of nodules, chlorophyll and N content of the plant, and amounts of total and fixed N decreased progressively with increasing salinity levels. In both non-saline and saline (50 and 100 mM NaCl) conditions, inoculations with *R. leguminosarum* bv. *Ciceri* strains isolated from wild chickpeas significantly increased all the symbiotic parameters when compared with the uninoculated control treatment. Garg and Sharma (2013) studied stress tolerant forms of rhizobia isolated from *Trigonella foenumgraecum*. Growth of isolates on yeast mannitol medium having variable range of pH (4-10) and different concentration of NaCl (0.05 - 5%) was determined. Among all isolates, four (RTF1, 2, 5, 10) were found salt tolerant. 5 isolates RTF1, 2, 3, 9 and 10 were pH tolerant and 6 isolates RTF1, 2, 3, 5, 7 and 8 were temperature tolerant.

Mesorhizobium isolates were divided into four major categories on ACC utilization pattern (Table 2). Several other bacterial strains that can utilize ACC as a sole source of nitrogen have been isolated from rhizosphere soil samples and subsequently used for inoculation purposes (Glick, 2003; Glick et al., 2007a, b, Govindasmay et al., 2008). Zafar et al. (2007) isolated twenty seven isolates of rhizobacteria containing ACC-deaminase from the lentil rhizosphere by using dilution plate technique. All the rhizobacterial isolates had the potential to improve the growth of lentil seedlings under axenic conditions. Khandelwal and Sindhu (2012) found that 38.9% *Pseudomonas* isolates obtained from cluster-bean rhizosphere showed good growth on ACC supplemented plates. These ACC utilizing rhizobacteria are potentially important for agricultural practice and rhizobial or *Pseudomonas* strains that are intended for use as inoculants of host legumes should first be selected/ tested for the presence of a functional ACC deaminase.

Higher level of ethylene which is applied either directly or indirectly had significant inhibitory effect on nodulation (Guinel and Sloetjes, 2000). Inoculation with ACC deaminase containing *Rhizobium* had the potential to improve plant growth by reducing the inhibitory effect of salinity. Single inoculation have shown positive response to the measured growth parameters that might be attributed to changes in endogenous ethylene level by presence of plant growth promoting bacteria containing ACC-deaminase on the roots of legumes (Shahroona et al., 2006; Nadeem et al., 2009; Ahmad et al., 2011). Compared to uninoculated plants, nodule number, nodule fresh and dry weight was considerably improved by sole inoculation of ACC^+ *Mesorhizobium* isolates (Tables 3 and 4). Shahzad et al. (2010) reported that inoculation with selected isolates increased the root length, shoot length, dry root weight, shoot dry weight, lateral root number, lateral root length and lateral root dry weight of chickpea seedlings up to 107.5, 57.4, 86.7, 83.5, 266.7, 286.6 and 121%, respectively, over uninoculated control plants. Similar enhanced nodulation and increase in plant biomass production has been reported by inoculation of legumes with different rhizobial strains (Belimov et al., 2002; Dey et al., 2004; Mayak et al., 2004a). These ACC^+ *Mesorhizobium* sp. *Ciceri* strains having the ability to improve root nodule mass and shoot biomass could be further tested for symbiotic effectiveness under pot house and field conditions. Furthermore, the use of rhizobial strains with ACC deaminase activity might be very important for developing microbial inocula for agricultural purposes.

Conflict of interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Effects of gelatin coating glass coverslips on fungal attachment and their morphological demonstrations

Supattra Suwanmanee[1] and Natthanej Luplertlop[2]*

[1]Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand.
[2]Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand.

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Gelatin obtained from collagen, an extracellular matrix (ECM) protein, plays a crucial role in facilitating cell attachment. This study aimed to determine the effects of gelatin on fungal attachment and morphology. Samples of *Aspergillus niger* were grown on gelatin-coated coverslips and non-gelatin-coated coverslips, and prepared for examination under both light and scanning electron microscopes. The results show that the attachment and morphology of *A. niger* grown on gelatin-coated coverslips differ from that grown on non-gelatin-coated coverslips. *A. niger* grown on gelatin-coated coverslips exhibited higher levels of attachment, and less evidence of fungal detachment and dissemination during histological process than non-gelatin-coated coverslips. In addition, gelatin-coated coverslips presented more complete structures of fungi and better image quality than non-gelatin-coated coverslips. It is suggested that gelatin coating may be used in fungus slide preparation to prevent fungal damage during experiments, at the same time improving image quality.

Key words: Attachment, coverslip, fungus, gelatin, morphology.

INTRODUCTION

The extracellular matrix (ECM) is the material found on the outside of cells. It typically provides structural and biochemical support to surrounding cells. ECM is composed of a variety of proteins and polysaccharides. ECM proteins play a crucial role in supporting cell attachment and growth. One of the most prominent ECM proteins is collagen (Engler et al., 2006; Frantz et al., 2010).

Gelatin is a translucent, colorless, odorless, and tasteless solid substance, derived from collagen, which is one of the most well-known ECM proteins in animals. It is a denatured protein obtained either by partial acid hydrolysis of pig skin type I collagen (gelatin type A) or alkaline hydrolysis of bovine collagen (gelatin type B) (Kommareddy et al., 2005; Mohanty et al., 2005). It does not produce toxic byproducts. Therefore, it is a very suitable protein for use in many different applications. A previous report found that gelatin is used clinically as a plasma expander, as well as a stabilizer in vaccines (Kommareddy et al., 2005; Elzoghby et al., 2012). The results from an *in vivo* study in rats demonstrated that gelatin can be used in wound care as a wound healing agent (Kale et al., 2011).

The use of gelatin in biological applications is becoming increasingly widespread. Gelatin contains informational signals such as tripeptide sequence Arg-Gly-Asp (RGD), which modulates cell adhesion, migration, differentiation, and proliferation, thereby improving the final biological

*Corresponding author. E-mail: natthanej.lup@mahidol.ac.th. Tel: 66-02 306-9172. Fax: 66-02 643-5583.

behavior of cells (Sakai et al., 2009; Tan and Marra, 2010; Wang et al., 2012). Many species of fungi have structures that are fragile such as hyphae and conidia. During the microscopic method with conventional fungal slide culture, these fragile structures may extremely be prone to damage and slip off even if they are handled with care. Incomplete structures of fungi in microscopic slides are difficult to identify and can cause misinterpretation and diagnostic error. To overcome this problem, new techniques to improve fungal slide cultures will be explored. Previous data found that gelatin is the most frequently used medium for histological purposes. To preserve tissue sections on histological slides during the staining and washing steps, slides need to be coated with adhesive substances. Gelatin-coated slides can enhance tissue section attachment. Gelatin-coated surfaces can decrease section detachment in staining and washing steps (Frost et al., 2001). Therefore gelatin may promote adhesion of fungal on culture slide and prevent the destruction of fungi during the slide preparation.

There are many reports on the use of gelatin in biological applications. However, data on the benefits of using gelatin, in regard to fungal attachment and morphology, is still lacking. Therefore, the purpose of this research was to examine the effects of gelatin on fungal attachment and morphology via light and electron microscopy.

MATERIALS AND METHODS

Fungal strain and growth conditions

A. niger were used in this experiment because they have fragile structures that are extremely prone to damage, and can slip off even when handled with care. *A. niger* is also an opportunistic pathogen and is the most common fungi found in contaminated environment. In addition, they grow easily and fast on agar. The *A. niger* used in this research were obtained from the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Thailand. They were cultured in Sabouraud dextrose agar (SDA), SDA plus yeast extract (SDA+YE), and trypticase soy agar (TSA) (Difco Laboratories, Detroit, MI, USA) prior to the study.

Preparation of 10% gelatin-coated coverslips

The 10% gelatin solution was prepared by dissolving gelatin (Sigma-Aldrich Co., St. Louis, MO, USA) in autoclaved deionized H_2O. The sterilized 10 mm round coverslips were placed into the wells of a sterile 12-well plate (one coverslip per well). Then gelatin solution was added in each well to cover each coverslip. After the coverslips were incubated for 10 min at room temperature, the gelatin solution was removed. The coverslips were allowed to air dry in a laminar flow hood under UV light for at least 2 h. The coated coverslips can be stored at room temperature for further use.

Coverslip culture

Agar plugs of *A. niger* were inoculated onto agar in 6-well plates (Sample number (n) = 30 per type of agar, three replicates for each experiment). The 10% gelatin-coated coverslips and non-gelatin-coated coverslips were placed separately either over or under the inoculated fungi. Fungal cultures were incubated at 25-30°C for 3 days to allow the fungi to grow on coverslips before morphological examination.

Morphological studies

Preparation for light microscopy

The coverslip culture was wetted on the specimen side with a drop of 90% ethanol. One drop of lactophenol cotton blue was applied to the specimen, before the coverslip culture was gently lowered onto a drop of lactophenol cotton blue on a glass slide. The slide was left to dry and then sealed with Permount. For hematoxylin and eosin (H&E) staining, the coverslip culture was dipped in 100 and 95% ethanol, rinsed in distilled water, and stained with hematoxylin and eosin (Ajello et al., 1963). Fungal morphology was examined under a light microscope (Axio Lab.A1; Carl Zeiss, Oberkochen, Germany).

Preparation for scanning electron microscopy

The coverslip culture was mounted on an aluminum stub, coated with a thin layer of gold particles using a sputter coater (K550; Emitech, Kent, England), and observed under a scanning electron microscope (JSM-6610LV; JEOL, Tokyo, Japan) (Bozzola and Russell, 1992).

RESULTS

After the preparation of fungal coverslip culture (Figure 1a-c), the two groups of coverslips were observed, comparing fungal attachment and morphology. There was no significant difference in fungal density between gelatin-coated coverslips and non-gelatin-coated coverslips independently by the fungal agar media employed (data not shown). The results show that the attachment and morphology of *A. niger* grown on gelatin-coated coverslips differed from those grown on non-gelatin-coated coverslips, as described below.

A. niger stained by lactophenol cotton blue (Figure 2a, b) and H&E (Figure 2c, d) were observed under a light microscope. The fungal attachment on non-gelatin-coated coverslips (Figure 2a, c) was less efficient, with a higher rate of detachment during the staining and washing steps than with gelatin-coated coverslips (Figure 2b, d). In the case of the non-gelatin-coated coverslips, incomplete structures of *A. niger*, such as broken conidiophores, dispersed conidia, or lost conidia were observed. Some structures of *A. niger* on non-gelatin-coated coverslips, such as conidia or vesicles, were stained as pale color or colorless. Some areas of the image had multiple layers. Some areas were blurred and out of focus, resulting in the loss of image detail. When using gelatin-coated coverslips, there were fewer incomplete fungal structures.

There were no pale colored or colorless specimens in the samples. The fungi were brightly colored. In addition, images showed clear details of the structure of the fungi, such as conidia. These results were confirmed by scanning electron microscopy. The electron microscopic results were similar to the light microscope.

Gelatin-coated coverslips showed more complete

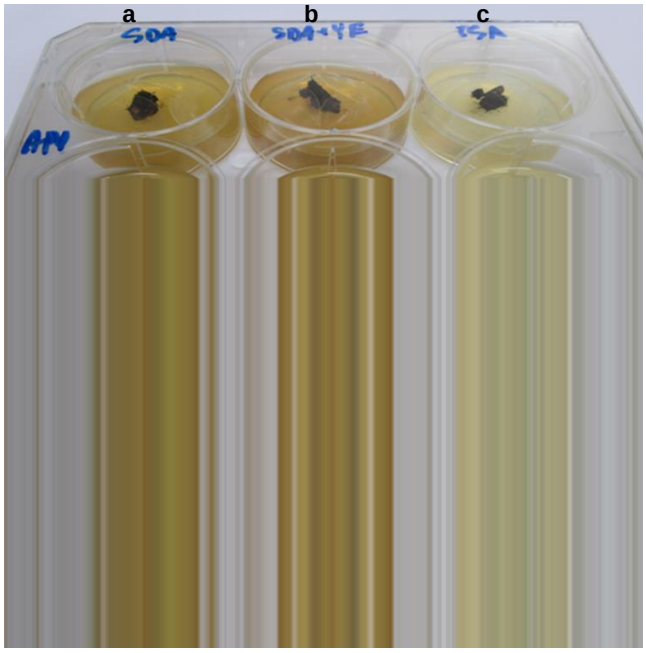

Figure 1. Sequence of preparation of fungal coverslip culture. (a) Samples of *A. niger* were inoculated on fungal media and then coverslips were placed on the inoculated fungi. (b) Fungi started to grow on media. (c) After 3 days of incubation, fungi grew out onto a part of the coverslips.

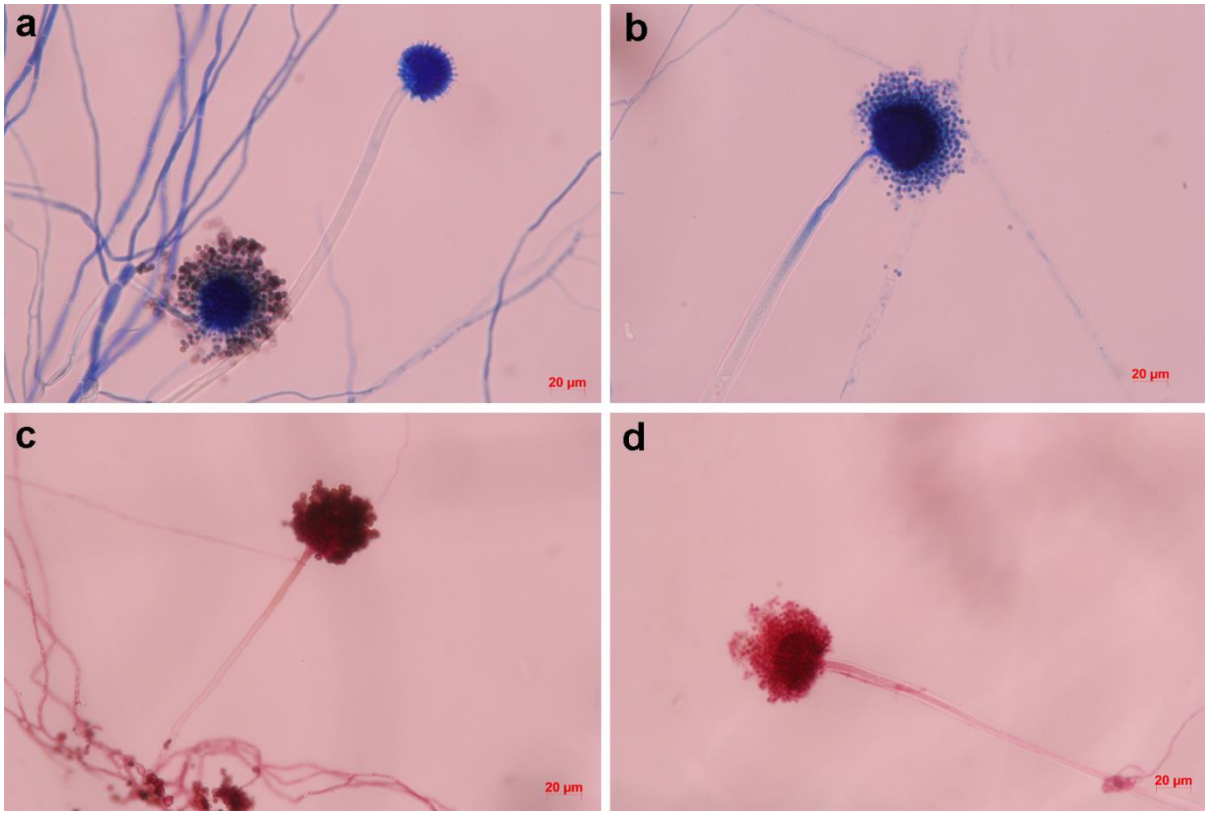

Figure 2. Light micrographs from lactophenol cotton blue (a, b) and H&E (c, d) staining of *A. niger* formed on different types of coverslips. (a, c) Non gelatin-coated coverslips, showing incomplete structure of *A. niger* (a) and blurred image (c). (b, d) Gelatin-coated coverslips, demonstrating complete structure of *A. niger*, with clear details of image (×40).

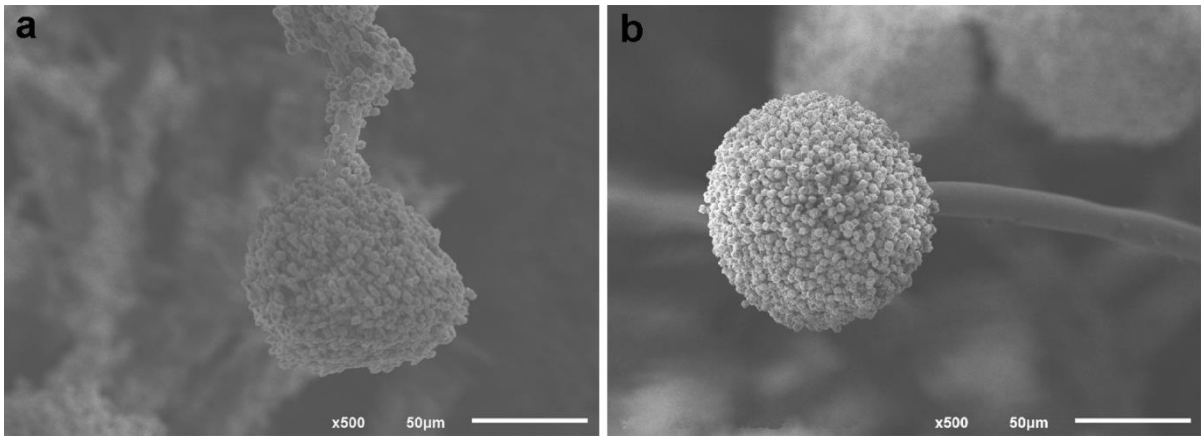

Figure 3. Scanning electron microscope images of *A. niger* grown on different types of coverslips. (a) Non gelatin-coated coverslip, demonstrating out of focus image. (b) Gelatin-coated coverslip, illustrating complete structure of fungus with clear details of image.

structures of *A. niger* and provided more detailed images (Figure 3b). In contrast, higher numbers of incomplete structures of fungi and blurred images were observed in non-gelatin-coated coverslips (Figure 3a).

All the experiments were conducted in triplicate. The quality of the results including attachment and morphology of *A. niger* achieved by growing on gelatin-coated coverslips was better than non-gelatin-coated coverslips. 73% of the total fungal slides of gelatin-coated coverslips could be interpreted, compared with 48% of the total fungal slides of non-gelatin-coated coverslips.

DISCUSSION

Many species of fungi form very delicate structures which are at least partially destroyed by even the most careful hands. It is very difficult to prepare slide cultures of fungi for microscopic examination. Many researchers have modified a variety of slide culture methods to solve this problem (Reiss, 1970; Mitchell and Britt, 1981; Ellis and Ajello, 1982; Harris, 1986; Fujita, 2013). In this study, a new culture method was applied, which prevented fungal damage during the experiment. This method permitted the growth of fungi on the coverslips. It is known that most cell types can be grown on soft materials, but in this study, *A. niger* started growing on agar (SDA, SDA+YE, TSA), and was then allowed to grow on a rigid substrate composed of coverslips coated with a thin film of gelatin.

The results of the study revealed that gelatin can promote the attachment of *A. niger* to coverslips and reduce the loss of samples of *A. niger* from coverslips during histolo-gical processes. Moreover, gelatin-coated coverslips showed more complete fungal structures and more detailed images than non-gelatin-coated coverslips.

Gelatin obtained from collagen, ECM protein, plays an important role in promoting cell attachment. The microscopic data of this experiment showed that gelatin can enhance the adhesion of fungi to coverslips. This finding was similar to previous reports of many cell types in culture. An *in vitro* study found that gelatin incorporated into poly(lactide-co-glycolide) (PLGA) nanofibers can promote the material surface properties for fibroblast cell attachment and proliferation (Hu et al., 2013). Gelatin combined with poly(3,4 ethylenedioxythiophene)-tosylate (PEDOT (TOS)) can support bovine brain capillary endothelial cell (BBCEC) adhesion and growth on well plates. BBCECs on gelatin/PEDOT(TOS)-coated well plates were elongated, which was a sign of initial adhesion, whereas BBCECs on other well plates remained round and non-elongated (Bongo et al., 2013). Cell adhesion is the binding of a cell to a surface or substrate, such as an ECM or other cell. Many factors are known to mediate adhesion between the cell and another surface or substrate. It is known that the substrate's surface morphology has an effect on adhesion, proliferation, and function of cells (Zhu et al., 2003). The data from a previous study found that the roughness values of gelatin and glass slide were 1.7±0.9 nm and 0.6±0.5 nm, respectively (Bongo et al., 2013). This increased roughness provides opportunities for greater fungal adhesion, as shown in this study. Therefore, gelatin can help prevent fungal damage during experiments, by increasing fungal attachment on coverslips and reducing the loss of fungi from coverslips during histological procedures.

A previous report found that filamentous fungi can form biofilms, complex 3D structures with cells usually enclosed within an extracellular matrix consisting of polymeric substances (EPS). A biofilm is characterized by cells that are securely attached to a surface and/or other cells within EPS (Stoodley et al., 2002; Harding et al., 2009; Siqueira and Lima, 2013). In this study, gelatin may stimulate EPS production or act as biofilm that result in the increase of the attachment of *A. niger* to coverslips.

In this study, lactophenol cotton blue, the most conventional stain for microscopic examination of fungi and H&E, the most conventional stain for light microscopy, were able to stain fungal cell wall. The results show that *A. niger* grown on gelatin-coated coverslips had more attach-ment and better morphology than that grown on non-gelatin-coated coverslips. The results were also confirmed by scanning electron microscopy. Our study demonstrates that lacto-phenol cotton blue and H&E staining were not significantly different, but no data exists concerning other staining methods, fungal attachment and morphology.

The data from this study is very beneficial for preparing fungal slides for histology. Gelatin is an alternative substrate which creates a surface which can hold fungi on coverslips more securely, therefore preventing the separation of fungi from coverslips. Gelatin makes coverslips rough for the fungi to adhere more strongly. This new technique helps to protect against misinterpretation and diagnostic error from incomplete structures of fungi in microscopic slides. Gelatin-coated coverslips can prevent the destruction of fungi during the experiment and improve image quality, making it easier to observe fungal cells. In contrast, blurred images resulted from using non gelatin-coated coverslips. Gelatin is also very easy to prepare, convenient, and relatively inexpensive. Prepared gelatin solutions can be kept for up to 1 month. Gelatin-coated coverslips can be stored in closed boxes at room temperature for several weeks. In addition, fungal slides using gelatin-coated coverslips can be stored for a longer time than non-gelatin-coated coverslips. This new technique is more easily performed and saves time.

Conflict of interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Antimicrobial activity of extracts from an endemic *Salvia cilicica* Boiss. and Kotschy

Nur Tan[1]*, Burcu Sen[1], Merve Bilgin[2] and Emir Tan[2]

[1]Department of Pharmacognosy, Faculty of Pharmacy, University of Istanbul, 34116 Beyazit Istanbul, Turkey.
[2]Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Yeni Yuzyil, 34010 Cevizlibag Istanbul, Turkey.

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The antimicrobial activities of the different aerial and root extracts of *Salvia cilicica* were determined aiming to evaluate whether, it can be used in phytotherapy as an antimicrobial agent. In this study, the antimicrobial activity of roots and aerial parts of *S. cilicica* extracts was evaluated using micro dilution and disc diffusion methods against Gram positive and Gram negative reference standard microorganisms and yeast *Candida albicans*. All of the extracts, with the exception of ethanol extract, showed antimicrobial activity by using minimum inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) tests. The roots-petroleum ether and acetone extracts showed the highest antimicrobial activity (MIC's ranges 20-313 µg/ml) against Gram positive bacteria. The root-acetone extract showed higher antimicrobial activity against Gram negative bacteria in comparison to other extracts. The most remarkable result of the antimicrobial activities is that, except for the ethanol extract, the *S. cilicica* had a good inhibitory effect (MIC's ranges 20-313 µg/ml) against *Bacillus cereus* and *Salmonella cholerasuis*, and was noticed to be more active in paper disc diffusion test against Gram positive than Gram negative bacteria. The roots petroleum ether and acetone extracts exhibited activity against *C. albicans*. In conclusion, *S. cilicica* had a potential therapeutic value supporting its traditional usage in folk medicine.

Key words: *Salvia cilicica*, Gram positive, Gram negative, yeast, antimicrobial, endemic.

INTRODUCTION

The genus *Salvia* L. (Lamiaceae) comprises about 900 species world-wide, while it is presented with 89 species and 93 taxons in Turkey, approximately half of which is endemic. The genus has been distributed extensively in 3 regions of the world: 500 species in Central and South America, 200 species in western Asia, and 100 species in eastern Asia (Davis, 1988a; 1988b; Walker and Sytsma, 2007). Anatolia is the major gene center in Asia. *Salvia* species are used in folk medicine for the treatment of a variety of diseases, including infectious diseases. The species of *Salvia*, known as ‘‘adacayi’’ in Anatolia, are used as antiseptics, stimulants, diuretics and for wound healing in Turkish folk medicine and for herbal teas (Baytop, 1999; Demirci et al., 2003; Tepe et al., 2005).

*Corresponding author. E-mail: nur.tan@gmx.de.

Since the antimicrobial and antioxidant activities of these species, especially *Salvia officinalis*, were determined, similar studies on these species increased gradually all over the world. These studies suggest that the hydroxyl-cinnamic acid analogs, flavonoids and diterpenoids contribute to the biological activities of the *Salvia* species (Deans and Simpson, 2000). The essential oil and various extracts of *Salvia tomentosa* Miller showed a moderate activity against *Staphylococcus aureus (S. aureus), Streptoccocus pneumoniae, Moraxella catarrhalis, Bacillus cereus (B. cereus), Acinetobacter lwoffii, Clostridium perfringens, Mycobacterium smegmatis* and *Candida albicans* (*C. albicans*) with water insoluble extracts (Tepe et al., 2005).

In a previous study, the extracts of the aerial parts of 16 *Salvia* species were tested against five isolates (*B. cereus, S. aureus*, *K. pneumoniae, E. coli)* and *M. tuberculosis)*. The extracts of the majority of species exhibited moderate to good antibacterial activity with minimum inhibitory concentration (MIC) values ranging from 0.03 to 8.00 mg/ml, while the highest activity elicited was that against *M. tuberculosis* (MIC<0.50 mg/ml) with *S. radula, S. verbenaca* and *S. dolomitica* (Kamatou et al., 2007).

Askun et al. (2009) tested the antibacterial activity of *Salvia fruticosa* Mill., *Salvia tomentosa* Mill., *Sideritis albiflora* Hub.-Mor. (endemic), *Sideritis leptoclada* O. Schwarz & P.H. Davis, (endemic), and *Origanum onites* L. against *Staphylococcus aureus*, *Staphylococcus epidermidis (S. epidermidis)*, *Enterococcus faecalis (E. faecalis)*, *B. cereus*, *E. coli*, *Salmonella typhimurium (S. typhimurium)*, *Enterobacter aerogenes (E. aerogenes)*, and *K. pneumoniae*. The best antibacterial activity (MIC 640 µg/ml) was that shown against *S. typhimurium* and *E. aerogenes* by *S. fruticosa, E. coli,* and *S. typhimurium, E. aerogenes* by *S. tomentosa; S. typhimurium,* and *E. aerogenes* by *S. leptoclada* and *S. typhimurium, E. aerogenes* and *S. epidermidis* by *O. onites,* respectively.

In another study on the essentials oil, ethyl acetate and ether extracts of *S. urmiensis* Bunge, a high antimicrobial activity was observed with the ethyl acetate extract against *B. subtilis, C. albicans* and with the ether extract against *K. pneumoniae and Saccharomyces cerevisiae* (Farjam, 2012).

Salvia cilicica Boiss. and Kotschy (SC), an endemic species, has only a limited number of studies in the literature regarding its chemical composition and biological activity. In our previous study, we have presented the antileishmanial, antioxidant and cytotoxic activities. We also dealt with the isolation and structure elucidation of the terpenoid compounds from the root extracts of SC, which is utilized in traditional medicine (Tan et al., 2002).

Antibiotic resistance is a long-evolved trait in prokaryotes. In Europe, 25, 000 people die every year from drug-resistant infections. In 2009, there were 440, 000 new cases of multidrug resistant (MDR) tuberculosis in 69 countries. These figures, and rising resistance levels observed in the global surveillance programmes, show that antibiotic resistance has reached a critical point, as human and economic cost escalate. Antibiotic resistance is an emerging global healthcare threat and today's armory of antibiotics is limited. For some pathogens, the choice of available drugs is now greatly reduced. Several factors such as (i) increasing mortality from infections caused by resistant strains, (ii) the strong link between resistant pathogens and increasing levels of hospital-acquired infections, and (iii) the escalating healthcare costs have placed antibiotic resistance at the top of the healthcare agenda (ECDC/EMEA Joint Technical Report, 2009). Therefore, the investigations on antimicrobial activity increased year after year and these studies were focused on discovering new antimicrobial agents, especially from plant sources.

The aim of this study was to determine the antimicrobial activities of the different aerial and root extracts of SC; to compare their activities and to answer whether, SC can be used in phytotherapy as antimicrobial agent.

MATERIALS AND METHODS

Plant material and extraction

The aerial parts and the roots of *S. cilicica* Boiss. and Kotschy (SC) were collected from Adana- Pozanti (Turkey), in September 2011 and identified by Assoc. Prof. Dr. Nur Tan (Istanbul). The voucher specimen has been deposited in the Herbarium of the Faculty of Pharmacy, University of Istanbul (ISTE 98085).

The dried and powdered aerial parts (SCA) and the roots (SCR) of SC (1 kg of each species) were extracted in a Soxhlet respectively with petroleum ether (PE), acetone (Ac), ethanol (EtOH). The extracts were concentrated *in vacuo*.

Biological assays

Microorganisms and media

The antimicrobial activity of the extracts was evaluated against Gram positive and Gram negative reference standard microorganisms; *S. aureus* ATCC 25923*, S. epidermidis* ATCC 12228*, E. faecalis* ATCC 29212*, P. aeruginosa* ATCC 27853*, E. coli* ATCC 25922*, K. pneumoniae* ATCC 4352*, B. cereus* ATCC 11778*, B. subtilis* ATCC 6633*, P.mirabilis* ATCC 7002*, S. cholerasuis* ATCC 14028) and yeast *C. albicans* ATCC 10231 from the Institute of Microorganisms Culture Collection of Istanbul Medical Faculty/Istanbul/Turkey.

Ciprofloxacin (Bayer Türk Kimya San. Istanbul /Turkey), Amphotericin B (Gilead Sciences Ilac, Istanbul/Turkey), Dimethylsulfoxide (DMSO- Merck) Tryptic soy agar (TSA) from GBL, Istanbul/Turkey, Mueller Hinton broth (MHB), Mueller Hinton Agar (MHA) from TitanBiotek, Istanbul/Turkey and RPMI1640 medium from İnterlab /Sigma, İstanbul/Turkey were purchased.

Antimicrobial assays

The extracts were dissolved in DMSO and the volume was adjusted

Table 1. MIC and MBC (µg/ml) values of the root and aerial parts extracts parts in petroleum ether (PE), acetone (Ac) and ethanol (Et-OH) of *Salvia cilicica* Boiss.and Kotschy.

***Salvia cilicica* (SC)**	**SC Root**			**SC Aerial**			**Ciprofloxacin**	**Amphotericin B**
	Petroleum ether	**Acetone**	**Ethanol**	**Petroleum ether**	**Acetone**	**Ethanol**		
	MIC*/MBC µg/ml**							
S. aureus	78*/156**	313	1250	625/2500	625/1250	1250/2500	< 2	ND***
S. epidermidis	78/156	78/156	2500	625	313/625	2500	< 2	ND
E. faecalis	20	20/78	625/2500	78/156	156	2500	< 2	ND
B. subtilis	20/40	156/313	313/1250	625/1250	313	1250/2500	< 2	ND
B.cereus	40/78	78	313/625	78/156	78/156	625/1250	< 2	ND
S. cholerasuis (typhi murium)	156	313	313	313	313	1250	< 2	ND
P. aeruginosa	1250/5000	156/1250	1250	625/2500	625/2500	625/2500	9/20	ND
P. mirabilis	1250/5000	313/625	625/1250	1250/2500	1250	313/625	2/4	ND
K. pneumoniae	78	20	78	78/156	40	40	< 2	ND
E. coli	313/625	625	625	625	625	625	< 2	ND
C. albicans	2500	1250	-	-	-	-	-	2

*MIC = minimum inhibitory concentration; **MBC = minimal bactericidal concentration; ***ND= not done

to 10000 µg/ml. The antimicrobial activity of the extracts was carried out under laminar air flow (Nüve mn 120) by using micro dilution method as described in M7A7/2006 for bacteria and M27A3/2008 for yeast, and disc diffusion method as described in M02 A11/2012 for bacteria and M44A2/2009 for yeast by Clinical and Laboratory Standards Institute (CLSI,Wayne, Pennsylvania USA). Minimum inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were determined for each extract. Ciprofloxacin (20 µg/ml) and Amphotericin B (20 µg/ml) were used as the positive control, respectively.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The inoculums were prepared by diluting overnight cultures in saline (approx.10^8 CFU/ml for bacteria and 10^7 CFU/ ml for *C. albicans*). Bacteria and yeast were grown overnight on nutrient agar and on sabouraud dextrose agar (SDA) plates, respectively. The inocula were prepared from overnight grown cultures (bacteria for 24 h 37°C and yeast for 24-48 h 35°C) and the formed turbidity was adjusted to 0.5 McFarland units approximately 10^8 CFU/ml for bacteria and 10^7 for yeast.

The MIC and MBC values of extracts were determined on the basis of microbroth dilution method in 96 multi-well microtitre plates. The 50 µl Müller Hinton Broth (MHB) for bacteria and 50 µl RPMI1640 for yeast were added to the wells starting from the second well and continuing up to the twelfth. The crude plant extracts of 10000 µg/ml were added to the first and second wells. Two-fold serial dilutions were made achieving a final concentration ranging from 5000-9.76 µg/ml. One row of positive controls for Ciprofloxacin (final concentrations from 512 to 1 µg/ml) and for Amphotericin B (final concentrations from 250 to 0.5 µg/ml) were added to each plate. In addition, an extra row of DMSO was used as a vehicle control to determine it's possible inhibitory activity.

After incubation (bacteria at 37°C for 24 h and yeast at 35°C for 24-48 h) the microtitre plates were examined visually for microbial growth which appeared as visible turbidity. In each row, the well containing the least concentration and showed no visible growth was considered the MIC. MBC and MFC were determined by taking samples from the wells which showed no visible growth. The bacterial samples were inoculated on TSA plates and incubated at 37°C for 24 h while the yeast sample was inoculated on Sabouraud plates and incubated at 35°C for 24-48 h.

Disc diffusion technique

Antimicrobial activity was determined using bacterial cultures adjusted to 0.5 McFarland turbidity standard and inoculated onto TSA plates (diameter: 15cm). *C. albicans* was adjusted to the concentration of 10^6 CFU/ml. Cultures of *C. albicans* were suspended in sterile solution of 0.9% normal saline and the cultures were inoculated onto sabouroud dextrose agar plates.

Sterile filter paper discs (5 mm diameter for both bacteria and yeast) were impregnated with either 20 µl of extract dilutions, 10 µl ciprofloxacin (each disc containing 5 µg) or 10 µl amphotericin B (each disc containing 10 µg).

Bacterial cultures were then incubated at 37°C for 24 h and yeast at 35°C for 24-42 h. The antimicrobial activity was determined by measuring the inhibition zone on each paper disc.

RESULTS AND DISCUSSION

The extracts of SCA and SCR showed varying antimicrobial activities against the reference standard bacteria and yeast. The antimicrobial activity was summarized in Table 1 for micro dilution method and in Table 2 for disc diffusion method. The experiments were performed in duplicate and the results were expressed as average values.

In general, all the extracts (except EtOH extracts) showed a significant antimicrobial activity via MIC and MBC tests, but especially SCR-PE and -Ac extracts showed the highest antimicrobial activities against the tested Gram positive bacteria, and SCR-Ac extract had

Table 2. Antimicrobial activity (zone of inhibition, mm) of various extracts *Salvia cilicica* Boiss. and Kotschy against reference strains.

Salvia cilicica (SC)	SC Root			SC Aerial			Ciprofloxacin	Amphotericin B
	Petroleum ether	Acetone	Ethanol	Petroleum ether	Acetone	Ethanol		
S. aureus	12 ± 0.28	9 ± 0.71	-	-	7 ± 0.63	-	33 ± 0.91	ND**
S. epidermidis	13 ± 0.14	11 ± 0.21	-	-	8 ± 0.63	-	30 ± 0.77	ND
E. faecalis	14 ± 0.71	12 ± 0.35	-	7 ± 0.49	9 ± 0.42	-	23 ± 0.98	ND
B. subtilis	15 ± 0.21	15 ± 0.28	-	-	11± 0.35	-	35 ± 0.21	ND
B. cereus	12 ± 0.57	11 ± 0.35	7 ± 0.56	8 ± 0.21	8 ± 0.49	-	32 ± 0.21	ND
S. cholerasuis	12 ± 0.28	10 ± 0.49	-	8 ± 0.49	9 ± 0.35	-	40 ± 0.42	ND
P. aeruginosa	-	-	-	-	-	-	30 ± 0.21	ND
P. mirabilis	-	11 ± 0.56	-	-	-	-	34 ± 0.91	ND
K. pneumoniae	10 ± 0.84	10 ± 0.63	-	7 ± 0.71	7 ± 0.49	6 ± 0.56	32 ± 0.42	ND
E. coli	-	8 ± 0.49	-	9 ± 0.98	9 ± 0.56	6 ± 0.35	40 ± 0.28	ND
C. albicans	10 ± 0.21	9 ± 0.35	-	-	-	-	-	26 ± 0.63

*(-) No measurable zone; **ND= Not done.

higher antimicrobial activity against the Gram negative bacteria in comparison to other extracts (Table 1). The SCR-Ac and SCA-Ac extracts showed MIC's ranges of 20-625 µg/ml and MBCs ranges of 20-1250 µg/ml against *S. aureus, S. epidermidis, E. faecalis, B. subtilis, B. cereus, S. cholerasuis* and *K. pneumoniae*. The SCR-PE extract resulted in MIC's ranges of 20-313 µg/ml and MBC's ranges of 20-625 µg/ml against *S. aureus, S. epidermidis, E. faecalis, B. subtilis, B. cereus, S. cholerasuis* and *E. coli*. However, these activities were weak in comparison to the positive control Ciprofloxacin. Furthermore, the SCA-EtOH and SCR-EtOH extracts showed MIC' and MBC's ranges 40-1250 µg/ml against *B. cereus, S. cholerasuis, K. pneumoniae* and *P. mirabilis*.

No antimicrobial activity of all extracts except SCR-Ac was observed against *P. aeruginosa.* The most remarkable result of the antimicrobial activities is that, all extracts of SC (except EtOH extracts) have a good inhibitory effect against *S. cholerasuis* and *B. cereus*. Hence no antifungal activity was observed against *C. albicans.*

The results of disc diffusion method are summarized in Table 2. The SCR-PE and SCR-Ac indicated significant antimicrobial activity against *E. faecalis, B. subtilis, S. aureus, S. epidermidis, S. cholerasuis, K. pneumoniae*, and yeast *C.albicans.* No antimicrobial activity of all extracts was observed against *P. aeruginosa* with the disc diffusion. However, all of the above mentioned activities were weak in comparison to the positive control Ciprofloxacin (23-40 mm zone) and Amphotericin B (26 mm zone). Overall, all extracts are more active in paper disc diffusion test against Gram positive bacteria than Gram negative bacteria. The PE and Ac extracts of SC-R showed the highest antimicrobial activity, followed by SCA-Ac and SCA-PE.

It has been estimated, that plants provide over 100,000 secondary metabolites, small-molecule compounds (Dixon, 2001) and for thousands of years medicinal plants have played a significant role in the treatment of a wide range of medical conditions, including infectious diseases. Some naturally occurring chemical compounds serve as models for a large percentage clinically proven drugs, and many are now being re-assessed as antimicrobial agents.

Haznedaroglu et al. (2001) have tested the antimicrobial activity of the essential oil of *Salvia tomentosa* and showed it remarkably inhibited the growth of tested Gram-positive (*S. aureus, S. epidermidis* and *E. faecalis*) and Gram negative (*E. Coli and E. cloacea*) bacteria except *P. aeruginosa.*

The antibacterial effect of essential oil of *Salvia heldreichiana* was shown against *E. coli, S. lutea* and *S. typhimurium*. In the same study, the oil of *S. cryptantha* inhibited the growth of *S. lutea* (Akin et al., 2010).

Karatas and Ertekin (2010) showed that all essential oils of *Salvia palaestina, Salvia multicaulis, Salvia syriaca* and *Salvia ceratophylla* possessed a good antibacterial activity against *B. subtilis, E. coli* and *S. aureus*, additionally essential oils of *Salvia multicaulis* possessed antibacterial activity against *P. aeruginosa* and essential oils of *Salvia syriaca* against possessed antibacterial activity *P.aeruginosa* and *S. pyogenes*.

Ibrahim et al. (2013) have tested methanol extracts of *Salvia libanotica* against *S.aureus* ATCC25923 standard strain and a clinical isolate of methicillin-resistant *S.aureus* (MRSA) and *S.libanotica* was at 4 mg/ml affective against MRSA.

To the best of our knowledge, the antimicrobial activity of *S. cilicica* has not been previously reported. In our study, generally all of the extracts, except EtOH, showed a significant antimicrobial activity via MIC and MBC tests. SCR-PE and SCR-Ac showed the highest activity against Gram positive bacteria, while the latter SCR-Ac extract

has proven to possess higher antimicrobial activity against Gram negative bacteria. Our results suggest that *S. cilicica* may be useful in the treatment of infectious diseases caused by *S. aureus, S. epidermidis, B. subtilis, B. cereus K. pneumoniae* and *S. cholerasuis*.

Conclusions

This is the first study to show the antimicrobial activity of SC and has opened up the possibility of the use of this plant in drug development for alternate therapy for the treatment of infections. However, further large-scale trials regarding on more pathogenic organisms, animal tests and toxicological investigations are required to provide more conclusive proof of their antimicrobial activity.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Detection of *Leptospira interrogans* in plasma and urine samples by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

Ramazan Rajabnia[1], Elaheh Ferdosi Shahandashti[2], Somayeh Yaslianifard[3], Elham Ghadami[4], Asefe Kheirandish[4] and Nafiseh Adhami[5]*

[1]Infectious Diseases Research Center, Babol University of Medical Sciences, Babol, Iran.
[2]Microbiology Department, Babol University of Medical Sciences, Iran.
[3]Department of Microbiology and Immunology, School of Medicine, Alborz University of Medical Sciences. Karaj ,Iran.
[4]Microbiology Department, Babol University of Medical Sciences, Iran.
[5]Islamic Azad University of Pharmaceutical Science, Tehran, Iran.

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Acute phase of leptospirosis has high mortality. A diagnosis can be designed with high sensitivity by testing blood and urine samples by polymerase chain reaction (PCR) for early diagnosis of this phase of disease. The aim of this research was to detect *Leptospira interrogans* by PCR technique in plasma and urine samples which were taken from symptomatic patients. Clinical isolates were obtained in hospitals of north Iran, and analyzed by PCR method and confirmed with restriction fragment length polymorphism (RFLP). DNA was extracted from plasma and urine samples and the quantity of *L. interrogans* DNA was determined by using PCR technique with LP1 and Lp2 primers. RFLP technique was used by Alu I restriction enzyme for confirming of detection of one of the prevalent serogroup of leptospira in Mazandaran Province. In this study, more than 90 blood and urine samples were examined, 10 (9%) blood and 3 (3%) urine samples became positive by molecular method. The high specificity and sensitivity of molecular assay provide valuable tools for the early diagnosis of acute leptospirosis.

Key words: Restriction fragment length polymorphism (RFLP), *Leptospira interrogans*, polymerase chain reaction.

INTRODUCTION

Leptospira is a genus of spirochete which causes disease. Leptospirosis is one of the most common zoonotic diseases which are common between humans and animals; this disease is common in tropical and subtropical humid areas of the world (Levett et al., 2005; Sharma and Kalawat, 2008;, Bharti et al., 2003; Ricaldi and Vinetz, 2006; Plank and Dean, 2000). All Leptospira serovars may cause Leptospirosis in humans. Some cases are without jaundice

*Corresponding author. E-mail: nafis.adhami@gmail.com. Tel: +989112248619.

(illness without jaundice) and some cases are with jaundice (Weil's disease, the jaundice form of the disease). Basically, animals get infected with Leptospirosis (Levett et al., 2005; Sharma and Kalawat, 2008).

Human infections are caused incidentally and subsequent to a contact with water or other materials which are infected with contami-nated secretions of host animals (Levett et al., 2005; Sharma and Kalawat, 2008; Bharti et al., 2003; Ricaldi and Vinetz, 2006; Plank and Dean, 2000; Park et al., 2006).

Those people who are exposed to contaminated water by voles (such as mine workers, farmers and fishermen) have the highest risk of infection. Human infection is mostly caused by drinking infected water or eating infected food. In rare cases, the organism may enter the body through mucous membranes or skin.

Leptospirosis is diagnosed based on identifying the patient's clinical picture and showing the antibodies against *Leptospira*. Isolating the bacteria from clinical samples through sample culture methods is very difficult, time consuming, lengthy and, most of the time, unsuccessful (Ricaldi and Vinetz, 2006; Park et al., 2006)

Human acute leptospirosis mostly is caused by infection with strains of the ictro haemorrhagiae serogroup of *L. interrogans* (Kee et al., 1994).

Pathogenic Leptospires are very fastidious and slow-growing and their isolation from clinical samples is very difficult and time consuming (Fonseca et al., 2006). In the first week of the disease, in which specific antibodies do not exist yet, serology is not useful; in this week, sample culture-serology methods and direct observation are not useful in early and rapid diagnosis of Leptospirosis.

Early diagnosis of leptospirosis is critical because of the risk of severe complications, such as pancreatic islet and vasculitis, as well as lung and intracranial haemo-rrhages, which require intensive care therapy.

In addition, leptospirosisis is usually misdiagnosed because of its variable and non-specific clinical symptoms that has over-laps with other febrile diseases, such as dengue fever or other haemorrhagic fevers (Fonseca et al., 2006). Detection of small numbers of leptospires in clinical samples has become practical due to specific polymerase chain reaction (PCR). This is important as leptospirosis can run a fulminant course and patients may die before the development of the characteristic clinical protests of leptospirosis or the appearance of leptospiral antibodies or both, and, therefore, the disease may go unrecognised. Post-mortem diagnosis may fail because leptospires may die before inoculation of culture medium and specific antibodies may not yet be demonstrable in serum samples.

Therefore early diagnosis of leptospirosis is important because severe leptospiral infection can run a fulminant course. The polymerase chain reaction (PCR) was evaluated for the detection of leptospires in clinical samples from patients with acute leptospiral infection ("Kee et al., 1994).

MATERIALS AND METHODS

Subjects and methods

In this study, blood and urine samples of 90 suspected patients, who were hospitalized based on clinical symptoms, were collected from May to late September in 2012. The blood and urine samples of the first and the second weeks of the disease were used for detection. All the patients were 60 men and 30 women. 4 ml of blood and 10 ml of urine samples were obtained from all suspected patients, who were admitted in the infectious diseases wards of Ayatollah Rohani Hospital in Babol and Razi Hospital in Gha'emshahr.

Plasma separation

The patients' bloods were poured into twisty glass flotation tubes (Isolab, Germany) containing EDTA. Then, they were centrifuged at 2556 Rcf (g) for 30 min after that, the separated plasmas were put into micro-tubes and placed in a -20°C freezer (Honarmand et al., 2005).

Urine separation

10 ml of patient's urine was poured into a sampling tube. 10 µl of for-malin was added to make the bacteria fixed and motionless. Then it was centrifuged at 2556 Rcf (g) for 30 min. After that, the supernatant liquid was thrown away and 500 µl PBS was added to the remaining sediment; then, it was well mixed with vertex and was placed in a -20°C freezer (Honarmand et al., 2005).

PCR analysis

High Pure PCR Template Preparation Kit (Roche from German company) was used to extract the bacterial DNA. 200 µl of each sample was used for this extraction.

Since the amount of the separated DNA was very little and it was not enough for PCR, the extraction method was carried out in this way: 600 µl of each sample was used in separated micro-tubes; we had three 200 µl micro-tubes and each of them was filled with separa-ted materials; when it was time for the stage of passing through a filter, these three micro-tubes were passed through the same filter; the rest of the operation was carried out according to the kit instruct-tions. LP1 and LP2 primer pairs are exclusively designed for such *L. interrogans*. The LP1 and LP2 primer pairs generate 274 base-pair sequences (Kee et al., 1994).

LP1 :5'- ATA CAA CTT AGG AAG AGC AT-3'
LP2 :5'- GCT TCT TTG ATA TAG ATC AA-3

To optimize the amplification conditions, the parameters that affect the PCR including buffer, primer concentrations, $MgCl_2$ concentration, and annealing temperature were checked. The final optimized PCR Reaction consists of 0.4 µM of each prime,3 µL dNTP (10 mM), 2 µL $MgCl_2$, 0.3 unit Taq polymerase (Metabion, Martinsried, Germany), 5 µM PCR buffer, and 5 µL of DNA template in total volume of 50µl with double distilled water. The cycling program was adjusted as follows: initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 48°C for 45 s and 72°C for 30 s, with final extension 72°C for 7 min in 1 cycle.

The PCR product was electrophoresed on 1/5% agarosegel con-taining ethidium bromide stainingfor 1 hour and then it was photo-graphed (Figure 1). Extracted DNA of *Leptospira interrogans,* used as the positive control, and water used as negative control. We used 100 bp molecular markers size (made by Fermentas Company) and

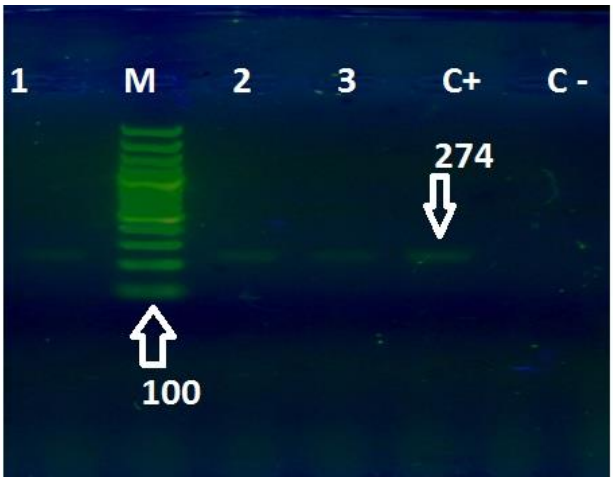

Figure 1. Gel electrophoresis. 1, 2, 3, Clinical positive sample. C^+, positive control, C^-, negative control.

Table 1. Detection of leptospires in human patients with polymerase chain reaction.

Sex	Sample	
	Plasma	Urine
Female	2 (2/2%)	0
Male	8 (8/9%)	3 (3/3%)
Total	10 (11/1%)	3 (3/3%)

thermo-cycler were amplification, respectively. To check the specificity of our primers used, several Gram positive and negative bacteria, such as DNA from *E. coli* (NTCC21157), *S. aureus* (ATCC29213), *E .feacalis* (ATCC292) *P. aeruginosa* (ATCC49189), *A. baumannii* (NTCC12156) and *K. pneumoniae* (NTCC5056), were used for PCR.

RFLP method was used to confirm the PCR production and to digest 274 bp sequences enzymatically. The RFLP method was performed according to the following protocol: 7 µl of deionized water, 2 µl of buffer, 10 µl of the PCR production, and 1 µl of Alu Ienzyme were mixed together. Then, it was placed in incubator (37°C) for 30 min to be incubated and the enzyme to be activated (Kee et al., 1994). After that, it was incubated at 65°C so that the enzyme gets disabled. Then, it was loaded on 2% agarose gel.

RESULTS

From the total of clinical specimens using primers LP1 and LP2, we had 13 (14/4%) positive case in PCR. For the results of 90 plasma samples, we had 10 (11.1%) and in 90 urine samples, we had 3 (3.3%) positive results in PCR detection (Table 1). On the other hand, one of the samples had plasma and urine positive PCR result. The above sample belongs to a patient with clinical and epidemiological history (Fever (39°C), respiratory insufficiency, blood icterus, thrombopenia, renal insufficiency, bathing in river). The remarkable thing in our result is that we have 11 positive cases in men and 2 positive samples in woman.

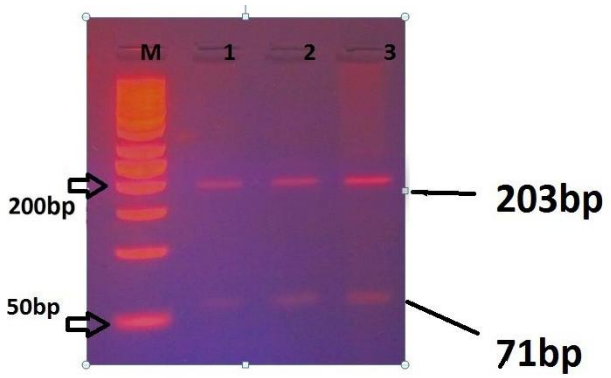

Figure 2. RFLP of positive samples in PCR results. M, ladder; 1, 2, 3, positive PCR sample.

In our study we used samples from 60 male and 30 female, therefore, our molecular analysis determined 18.3% leptospirosis in men group and 6.6% leptospirosis in women group. As a result of RFLP, Alu Ienzyme cleaved the sequence at two points, 203 and 71 bp and therefore confirms our PCR analysis (Figure 2).

DISCUSSION

Leptospirosis is a zoonotic disease with a worldwide distribution. Based on the receiving reports from different parts of our country regarding the increasing incidence of the disease and according to the importance of the health aspects of Leptospirosis (Bharti et al., 2003; Dezfully and Mehrabian, 2012), the study of rapid diagnosing methods of this disease is important.

Nowadays, culture and serological methods are used for diagnosing Leptospira; serological methods need at least one or two weeks and they cause humoral response in patients, and in culturing the mentioned period expands to several weeks (Organization, 2000; Guidugli et al., 2000; Vinetz, 2004; Merien et al., 1995; Turhan and Sezer, 2012; Brown and Levett, 1997).

PCR is being used for detection of a large number of microorganisms, including clinically important ones. The sensitivity of PCR is such that there will be no need for organism culture and separation. As a result, this method is the ideal method for rapid detection of the organisms involved in acute infections (Turhan and Sezer, 2012).

The practical value of PCR in diagnosing Leptospirosis is due to its ability in the detection of Leptospirain patients at the early stages. Leptospirosis is an acute disease and spreads quickly. Thus, its early and rapid diagnosis is very important in treatment.

It should be noted that applying sero-logical methods requires proper immunologic response and the passage of the course of the disease. On the other hand, the available serologic methods show the antibodies against Leptospira, but they cannot show Leptospira, while PCR

can show Leptospira even within the first days of infection.

In a study, Brown (1995) concluded that PCR can show Leptospira in acute stages of Leptospiral infection and it's a valuable method in diagnosing Leptospirosis at the early stages (Organization, 2000).

The inability of serological methods in rapid and accurate diagnosing of this bacterium has led to the consideration of PCR as an appropriate method due to its high sensitivity and accuracy. Since there is no sensitive diagnosing method for this bacterium and for determining its genotype in Mazandaran, this study was used as a rapid and accurate way of diagnosing and starting of a sensitive PCR technique. In this study, the isolated Leptospires were detected by PCR-RFLP as well.

In a study by Sun Huki in 1993 in Korea, the *liptospira interrogans* antibodies were detected by microscopic agglutination seven days after the infection. Then, the *L. interrogans* was detected by the use of LP2 and LP1 primers with 274 bp bonds (Kee et al., 1994).

In our study, it was detected by using LP2 and LP1 primers with 274 bp bonds, which were relevant to *L. interrogans,* as well. An important advantage of PCR-RFLP is that it can be applied directly to clinical samples, and immediately after the PCR of the clinical sample and the observation of the specified bond, RFLP can be implemented. Thus, the diagnosis will be approved as well (Savio et al., 1994; O'Keefe, 2002; Heinemann et al., 2000).

In comparison with Prolat's and Elis's studies, in our study, PCR was used for clinical samples and RFLP was implemented for approval. It seems that the negative cases of PCR belonged to those patients who were treated arbitrarily or tentatively. The response of Leptospira to antibiotic is as follows: liptospira escapes rapidly from the blood and migrates to Parenchymal tissue and high water content tissues, especially kidneys, lungs, liver and brain, and resides in there; thus, leptospiremia subsides and the number of bacteria of the blood decreases greatly (Brown et al., 1995; Perolat et al.,1990), since the Leptospira resulted bacteremia does not last more than 7-10 days in human and with the advent of antibody and treatment (antibiotics); the bacteria escape from the blood, thus there will be no bacteria and the problem of false-positivity of PCR is associated with other issues and mainly with saprophytic leptospira infection which are abundant in the environment (Turhan and Sezer, 2012; Organization, 2003).

This study shows that PCR can be effective in rapid diagnosis of the disease, but it is also likely to show false-negativity. In 90 patients suspected to Leptospirosis, only 13 of them had positive PCR; this may be due to the following reasons:

1) Leptospiremia subsided due to taking antibiotics. Some individuals of our sample had taken antibiotics.
2) Its low accuracy may be attributed to technical errors, inefficiency of the used chemicals, low amounts of DNA (or low amounts of bacteria) of the sample. In all these cases, PCR may generate false-negative responses.
3) The DNA extraction method and the kits which were used for this purpose may also affect the accuracy of PCR (Perolat et al., 1990).

Tony et al. (1997) and Savio et al. (1994) applied PCR-RFLP method to separate a large number of standard and isolated strains; they found it to be useful for rapid detection of subspecies of *L. interrogans* (Savio et al., 1994).

This study shows that men more than women are exposed to leptospirosis infection, perhaps it is for this reason that compared with women, most men work in agriculture and animal husbandry and generally can be stated that leptospirosis is a job-related illness.

In this study, PCR-RFLP was detected by LP2 and LP1 primers which were related to *Leptospira interrogans.* In our study, the evaluation criteria were very similar to the mentioned studies.

Conclusion

Of the 90 samples with primer LP1, LP2, 13 samples (4/14 of total) have been the piece. Thus, according to the high efficacy of PCR-RFLP method, it seems that it can be used directly for clinical samples and carry out the rapid diagnosis and identification simultaneously.

Conflict of interests

The authors have not declared any conflict of interest.

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