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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

Does *SEN* virus (*SENV*) infection affect the progression of chronic hepatitis C or B among Egyptian patients?

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The effect of *SENV* infection on chronic viral hepatitis is not very clear till now. Information regarding *SENV* infections in the Egyptian population where hepatitis viruses are prevalent is limited to a certain extent. We aimed to determine the frequency of *SENV* and its genotypes H and D in Egyptian patients with chronic hepatitis B (CHB) or C (CHC) and to study its possible role in the progression of liver disease. A total of 112 patients with chronic hepatitis (18 CHB and 94 CHC) were subjected to clinical assessment, laboratory and histological examinations. DNA from sera was extracted and *SENV* DNA was amplified by polymerase chain reaction. *SENV* DNA was detected in 28.6% of patients with chronic hepatitis (32/112). The percentage was 33.3 and 27.7% in CHB and CHC patients respectively. *SENV-H* was detected more frequently than *SENV-D* genotype. For CHC patients without cirrhosis (n=44), *SENV* was negative in 90.9% (40/44) compared to 9.1% (4/44) *SENV* positive giving a statistically significant difference (<0.001). A significantly higher percentage of patients showed twofold or more increase in the aspartate transaminase (AST) and alkaline phosphatase (ALP) among *SENV* negative CHC patients compared to *SENV* positive patients (P=0.04 , 0.03 respectively). In cirrhotic patients (n=58), both the levels of ALP and serum total bilirubin were significantly higher in *SENV* negative compared to *SENV* positive patients (P values were 0.01). For CHB patients, no statistically significant difference was detected regarding any of the studied parameters. We conclude that *SENV* does not worsen the progression of chronic viral hepatitis. This may reflect a possible protective effect of *SENV* in CHC patients which needs to be emphasized by further larger studies.

Key words: *SENV*, chronic, viral, hepatitis.

INTRODUCTION

The *SEN* virus (*SENV*) was considered to be a member of the family *Circoviridae*, genus Anellovirus, a group of non- enveloped, circular DNA viruses that also included the Torque teno (TTV) and its variants SANBAN,

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YONBAN, TUS01, and PMV (Sugiura et al., 2004). Now *Anelloviridae* is a highly divergent family of viruses that has three genera of anelloviruses capable of infecting humans: torque teno virus (TTV; *Alphatorquevirus*), torque teno minivirus (TTMV; *Betatorquevirus*), and torque teno midivirus (TTMDV; *Gammatorquevirus*) (Biagini, 2009).

SENV has a different geographic distribution and is fairly common around the world. Its prevalence has been found to vary in different populations (Tezcan et al., 2009). Phylogenetic analysis of *SENV* has demonstrated nine different genotypes: *SENV-A* to *SENV-I* which show 15-50% sequence diversities among them (Kojima et al., 2003). *SENV-H* and *SENV-D* genotypes were extremely associated with non-A to E hepatitis (Schroter et al., 2003).

Although not all transmission routes have been identified (Tezcan et al., 2009), yet it was reported that *SENV-D/H* could be transmitted by both parenteral and non-parenteral routes (faecal oral) (Umemura et al., 2001). Iatrogenic means in hospital setting is reported as a mode of transmission (Sagir et al., 1994). In addition, vertical transmission from mother to fetus does occur (Pirovano et al., 2002).

The prevalence of *SENV* has been investigated in patients with various forms of liver disease in many countries. *SENV* infection is frequently observed in patients with hepatitis B virus (HBV) (23 to 59%), hepatitis C virus (HCV) (22 to 89%), and in patients with hepatitis of unknown etiology (Schreter et al., 2006).

SENV may cause persistant infection that may exceed one year and has been documented as long as 12 years (Umemura et al., 2002). The role of *SENV* infection and the clinical significance were studied in patients with non A-E hepatitis or other viral hepatitis but the results are not very clear and are inconsistent to some extent and even show contradictory results (Wang et al., 2007; Cakaloglu et al., 2008). That is to say, coinfection with hepatitis B virus (HBV), or hepatitis C virus (HCV) has been reported to be associated with severe and progressive liver disease (Jardi et al., 2001). However, others reported that *SENV* was found to have no established pathogenicity and the exact role of this virus in the pathogenesis of liver diseases, including acute and chronic hepatitis, cirrhosis and the development of hepatocellular carcinoma (HCC) remains to be verified (Rizvi et al., 2013). Others suggested a protective role of *SENV* against HCV (Umemura et al., 2001). Information regarding *SENV* infections in the Egyptian population where HBV and HCV are prevalent is limited to a certain extent.

It has been stated that Egypt has the highest prevalence of hepatitis C worldwide and the epidemic will soon peak (Yahia, 2011). Overall, HCV prevalence among blood donors ranged between 5-25%, and among other general population groups between 0-40%. HCV prevalence among multi-transfused patients ranged between 10-55%, and among high risk populations reached up to 85% (Mohamoud et al., 2013). For HBV, the prevalence in Egypt was found to be 5% (Awadalla et al., 2011).

The aim of present study was to determine the frequency of *SENV* and its genotypes H and D in Egyptian patients with chronic hepatitis B (CHB) or C (CHC) and to study its possible role in the progression of liver disease in such patients.

MATERIALS AND METHODS

Study design

This cross sectional study was done in Assiut University Hospital after being approved by the Ethical Committee of the Faculty of Medicine, Assiut University. A verbal consent was also taken from the patients enrolled in the study.

Patients

A total of 112 patients with CHC or CHB were included in the study during a six months period (from June to December 2013). Patients were admitted to the department of Tropical Medicine and Gastroenterology, Assiut University. They were divided into 2 groups; patients with CHC (n=94) and CHB (n=18). None of the patients had a history of receiving antiviral therapy. For all patients, thorough clinical assessment, abdominal ultrasonography, laboratory tests and liver histopathology were done. The severity of cirrhosis was assessed by Child-Pugh classification (Child and Turcotte, 1964; Pugh et al., 1973)

We defined chronic hepatitis as persistent or intermittent elevation in alanine transaminase (ALT) or aspartate transaminase (AST) levels for more than 6 months with the presence of anti-HCV antibodies and positive serum HCV RNA for chronic hepatitis C (Anwar et al., 2006), positive HBsAg and positive serum HBV DNA for chronic hepatitis B and by liver biopsy showing chronic hepatitis with moderate or severe necroinflammation (Huntzinger, 2009).

Healthy controls

The control group included 20 healthy individuals of comparable age among which 12 (60%) were men and 8 (40%) women; their mean age being 45 years ± 3.6. These individuals were negative for HBsAg and anti-HCV.

Laboratory tests

Five milliliters blood was withdrawn from each subject under complete aseptic conditions. Sera were separated and stored frozen at -20°C until analysis. For all serum samples, the following laboratory tests were performed: Liver function tests (aspartate-aminotransferase (AST), alanine transaminase (ALT), Alkaline phosphatase (ALP), albumin, bilirubin and prothrombin time); Serological tests for HBV and HCV infection were determined by the ARCHITECT system for anti HCV and HBs Ag which is a chemiluminescent microparticle immunoassay (CMIA) for the qualitative detection of antibodies to hepatits C virus (Anti-HCV) and HBs Ag in human serum and plasma (Abbott GmbH, Wiesbaden-Delkenheim, Germany); HBV DNA was quantified by Real time PCR using artus HBV TM PCR kit (Applied Biosystems) and HCV RNA was quantified by Taq Man Assay Reagents

Table 1. Frequency of *SENV* in patients with chronic hepatitis.

Parameter	CHC (N=94)	CHB (n=18)	Controls** (n=20)
	***SENV* positive (%)**	***SENV* positive (%)**	***SENV* positive (%)**
SENV	26 /94 (27.7%)	6/18 (33.3%)	3/20 (15%)
SENV-H	10/26 (38.5%)*	2/6 (33.3%)*	0 (0%)
SENV-D	4/26 (15.4%)*	1/6 (16.7%)*	3/3 (100%)*
SENV-H/D	3/26 (11.5%)*	1/6 (16.7%)*	0 (0%)*
Negative for *SENV-H* or *D*	9/26 (34.6%)*	2/6 (33.3%)*	0 (0%)*

*The percentage was calculated against the number of *SENV* positive cases. ** P value between *SENV* positive controls and chronic hepatitis = 0.21.

(Applied Biosystems) using the 7500 fast Real Time PCR system; Polymerase chain reaction for *SENV* DNA.

Extraction of *SENV* DNA from serum

DNA was extracted from 200 µL serum using QIAamp DNA blood mini kit (Qiagen, Cat. No. 51104-Germany) according to the manufacturer's instructions

Amplification and detection

SENV DNA (349 bp) was detected by PCR according to Kojima et al. (2003). *SENV* common primers were used and were as following: forward primer AI-1F (5'-TWC YCM AAC GAC CAG CTA GAC CT-3'; W = A or T, Y = C or T, M = A or C), and reverse primer AI-1R (5'-GTT TGT GGT GAG CAG AAC GGA-3'). A 25 µl PCR mixture was used and consisted of: PCR master mix (12.5 µl), forward primer (AI – 1F) (0.5 µl), reverse primer (AI – 1R) (0.5 µl), distilled water (3 µl), extracted DNA (8.5 µl). Amplification was performed for 40 cycles, each included denaturation at 94ºC for 30 s, annealing at 52ºC for 30 s and extension at 72ºC for 60 s. This was followed by a final extension at 72ºC for 10 min to complete strand synthesis.

SENV-D DNA (231 bp) and *SENV-H* DNA (230bp) were detected by PCR with *SENV* specific primers, as previously described (Kojim et al., 2003). Type-specific primers D10S and L2AS and primers C5S and L2AS were used for *SENV-D* and *SENV-H* detections, respectively (Kojima et al., 2003). PCR mixture of 25 µl consisted of: PCR master mix (12.5 µl), forward primer (D10S) for *SENV-D* or (C5S) for *SENV-H* (0.5 µl), reverse primer (L2AS) (0.5 µl), distilled water (3 µl), extracted DNA (8.5 µl). PCR conditions for *SENV-D* and *SENV-H* genotypes were the same. Amplification was performed for 40 cycles, each included denaturation at 94ºC for 30 s, annealing for *SENV-D* at 58ºC for 30 s, for *SENV-H* at 50ºC for 30 s and extension at 72ºC for 60 s. Then, 10 min final extension at 72ºC was used to complete strand synthesis. PCR was performed in a DNA thermal cycler (HYBAID-PCR Express). The PCR products were separated using 1.5% agarose gel, stained with ethidium bromide, and visualised under a UV illuminator.

Liver histology

Liver biopsies were taken percutaneously with a 1.4 mm diameter Menghini needle and consisted of 3-5 mm long liver tissue cores. Biopsies were promptly fixed in 10% formalin, processed and embedded in paraffin blocks. Four µm sections were cut and slides were staind by hematoxylin-eosin and reticulin stains using the standard techniques. Modified hepatitis activity index (METAVIR) grading and staging were determined for each case according to the scheme given by Ishak et al. (1995).

Statistical data analysis

The statistical analysis was performed using statistical package for social sciences (SPSS) version 17.0 for Windows (SPSS, Chicago IL, U.S.A). Continuous data were expressed as means ± standard deviation (SD) and compared using Student's T test. Categorical variables were expressed as percentage and compared using chi-square ($\chi 2$) test and Fisher's exact probability test. A P value of ≤ 0.05 was considered significant.

RESULTS

Chronic HCV patients (n= 94) included 74 males and 20 females with a mean age of 47.2 ± 10.9 and chronic HBV patients (n=18) included 10 males and 8 females with a mean age of 48.4 ± 12.8. Cirrhosis was found in 50 patients with CHC (53.2%) and in 8 patients with CHB (44.4%). *SENV* DNA was detected in 28.6% of patients with chronic liver disease (32/112) and in 15% (3/20) of the control group but without a statistical significant difference (P = 0.21).

Table 1 shows the frequency of *SENV* and its genotypes H/D among the studied groups. *SENV* was detected in 27.7% of CHC cases (26/94) and in 33.3% of CHB patients (6/18). The commonest genus identified in both groups was *SENV H* in 38.5 and 33.3% respectively. However, *SENV D* was the only genus identified in controls (100%).

Combined *SENVH /D* were found in patients groups not amongst the controls. Analysis of PCR products for the presence of *SENV-H* and *SENV-D* DNA on agarose gel is shown in Figures 1 and 2.

On comparing the characteristics of patients with chronic hepatitis in relation to *SENV* viraemia (Table 2), we found that among patients with CHC without cirrhosis (n=44), *SENV* was negative in 90.9% (40/44) compared to 9.1% (4/44) *SENV* positive that was highly statistically significant (P < 0.001). On the other hand, no significant differences were found between *SENV* positive and negative CHB patients regarding any of the studied characteristics.

Regarding markers of severity of disease, we found that among patients with CHC without cirrhosis, the percentage of patients showing twofold increase in AST and ALT were significantly higher in *SENV* negative

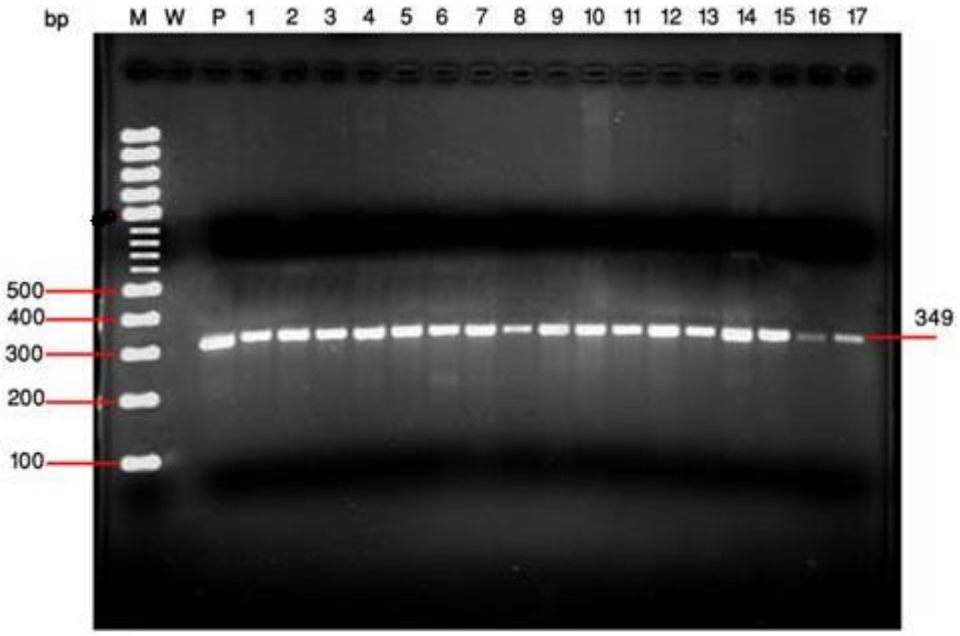

Figure 1. Detection of *SENV* DNA. Lane M, DNA 100 bp ladder; Lane W, negative control; Lanes 1- 17, positive cases in serum.

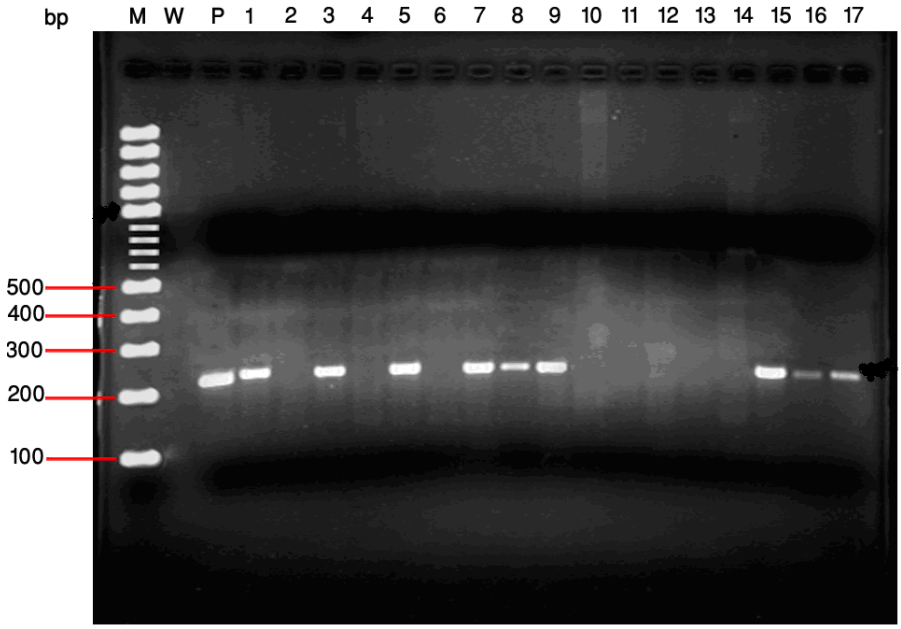

Figure 2. Detection of *SENV-D* / *H* DNA. Lane M, DNA 100 bp ladder; Lane W, negative control; Lane P, Positive control for *SENV* H; Lane 1, Positive control for *SENV* D; Lanes 2, 4, 6, 10, Negative samples for *SENV* D; Lanes 3, 5, 7-9, Positive *SENV* D; Lanes 11-14, Negative for *SENVH*; Lanes 15-17, Positive *SENV* H; Lanes 9, 17, were from the same patient.

compared to *SENV* positive patients (p=0.04 and 0.03 respectively). All the remaining clinical, laboratory and histological features were not of statistical significant difference as shown in Table 3.

Table 2. Characteristics of patients with CHC and CHB in relation to *SENV* viraemia.

Parameter	CHC patients (n = 94)				CHB patients (n = 18)			
	N	*SENV* positive (n = 26)	*SENV* negative (n = 68)	P	N	*SENV* positive (n = 6)	*SENV* negative (n = 12)	P
Age (mean±SD)	-	46.5 ± 15.9	47 ± 8.8	NS	-	51.3 ± 14.5	47 ± 12.3	NS
Sex								
Male	74	20 (27%)	54 (73%)	NS	10	4 (40%)	6 (60%)	NS
Female	20	6 (30%)	14 (70%)		8	2 (25%)	6 (75%)	
Duration of disease (years) (mean±SD)	-	3.6 ± 1.1	3.4 ± 1.5	NS	-	4.2 ± 2.2	3.4 ± 1.3	NS
History of blood transfusion (N)	8	8 (100%)	0	NS	0	0	0	-
Nature of liver disease								
Chronic hepatitis	44	4 (9.1%)	40 (90.9%)	< 0.001	10	4 (40%)	6 (60%)	NS
Liver cirrhosis	50	22 (44%)	28 (56%)	NS	8	2 (25%)	6 (75%)	NS

Table 3. Clinical, laboratory and histological features of CHC and CHB patients (without cirrhosis) in relation to *SENV* infection.

Parameter	CHC patients (N = 44)				CHB patients (N =10)			
	N	*SENV* positive (n = 4)	*SENV* negative (n = 40)	P	N	*SEN V* positive (n = 4)	*SENV* negative (n = 6)	P
Jaundice (n)	6	2 (33.3%)	4 (66.7%)	NS	2	0	2 (100%)	NS
Laboratory findings								
AST level[a]								
Mean IU/L ± SD	-	47 ± 32.6	87 ± 57.9	NS	-	76.4 ± 34.2	97 ± 78.5	NS
Elevated AST *	39	2 (5.1%)	37 (94.9%)	**0.04**	9	4 (44.4%)	5 (55.5%)	NS
ALT level[b]								
Mean IU/L ± SD	-	26.4 ± 18.9	50 ± 33	NS	-	42.8 ± 7.2	34.5 ± 12.8	NS
Elevated ALT *	32	1 (3.1%)	31 (96.9%)	**0.03**	0	0	0	-
ALP level[c]								
Mean (IU/L) ± SD	-	57.5 ± 20.2	83.2 ± 30.6	**NS**	-	74.9 ± 40	77.3 ± 33.6	NS
Elevated ALP *	4	0	4 (100%)	NS	0	0	0	NS
Albumin g/dl	-	3.2 ± 1.1	4 ± 0.7	NS	-	4.2 ± 0.4	4.3 ± 0.3	NS
Bilirubin mmol/l	-	26.8 ± 12.2	18.9 ± 10.7	NS	-	16.2 ± 5	19.7 ± 5.4	NS
Prothrombin time	-	12.6 ± 1	12.64 ± 1	NS	-	12.9 ± 1	12.5 ± 1.5	NS
Viremia (mean ± SD, log10 copies/mL)	-	5.91 ± 2.1	6.42 ± 1.2	NS	-	6.01 ± 1.46	6.68 ± 1.5	NS
METAVIR stage (n)								
1	22	0 (0%)	22 (100%)		2	2 (100%)	0	
2	14	2 (14.3%)	12 (85.7%)	NS	4	0	4 (100%)	NS
3	5	1 (20%)	4 (80%)		4	2 (50%)	2 (50%)	
4	3	1 (33.3%)	2 (66.7%)		0	0	0	
Metavier activity (n)								
A1	22	1 (4.5%)	21 (95.5%)		6	2 (33.3%)	4 (66.7%)	NS
A2	22	3 (13.6%)	19 (86.4%)	NS	4	2 (50%)	2 (50%)	

[a]Normal level: 0-32 IU/L; [b] Normal level: 0-45 IU/L; [c] Normal level: 30-120 IU/L; *Twofold or more.

Concerning cirrhotic patients, the level of ALP and serum total bilirubin were significantly higher in patients without *SENV* viraemia compared to patients with *SENV* infection (p values were 0.01 for both). Other parameters

Table 4. Comparison between cirrhotic patients with and without *SENV* infection regarding laboratory findings, severity of liver disease and liver cirrhosis related complications.

Parameter	*SENV* negative cirrhotics (N=34)	*SENV* positive cirrhotics (N=24)	p
Cause of cirrhosis			
HCV (50)	28 (56%)	22 (44%)	NS
HBV (8)	6 (75%)	2 (25%)	
Laboratory findings (mean±SD)			
AST (IU/L)	95.1 ± 61.3	118.2 ± 66.4	NS
ALT (IU/L)	52.9 ± 29.01	41.22 ± 22.5	NS
ALP (IU/L)	132.5 ± 67.6	99.4 ± 24	**0.01**
Serum albumin (g/dl)	2.2 ± 1.1	2.2 ± 0.6	NS
Serum total bilirubin (mmol/l)	90 ± 68.6	39.2 ± 20.4	**0.01**
Prothrombin time (seconds)	19.9 ± 7	17.3 ± 3	NS
Child-Pugh score	10.8 ± 2.7	10.4 ± 2.2	NS
Child-Pugh class			NS
Class A (5)	3 (60%)	2 (40%)	
Class B (7)	5 (71.4%)	2 (28.6%)	NS
Class C (46)	26 (56.5%)	20 (43.5%)	
MELD score	16.6 ± 8.9	13.7 ± 6.6	NS
Hematemesis (10)	6 (60%)	4 (40%)	NS
HE (14)	9 (64.3%)	5 (35.7%)	NS
SBP (19)	10 (52.6%)	9 (47.4%)	NS
HRS (18)	10 (55.6%)	8 (44.4%)	NS
HCC (14)	6 (42.9%)	8 (57.1%)	NS

HE, Hepatic encephalopathy; SBP, spontaneous bacterial peritonitis; HRS, hepatorenal syndrome; HCC, hepatocellular carcinoma.

regarding laboratory findings, severity of liver disease using Child-Pugh classification and liver cirrhosis related complications showed no significant differences (Table 4).

DISCUSSION

Patients with *SENV* infection develop a persistent infection that exceeds one year in approximately 45% and has been documented as long as 12 years (Umemura et al, 2002). The exact interaction of *SENV* with HCV and HBV is unclear (Tahan et al., 2003). *SENV-H* and D genotypes have been found at various rates in different populations and the role of *SEN-V* regarding the pathogenesis of liver disease is not yet known (Mu et al., 2004).

In the present study, *SENV* was detected in a considerable percentage of chronic hepatitis patients with genotype H being the most prevalent. We reported *SENV* DNA in 28.6% of patients with chronic liver disease (27.7% in CHC, 33.3% in CHB) and in 15% of the control group (3/20). The percentage of *SENV* among CHC patients is generally in accordance with the average of percentages recorded in different regions in Egypt that ranged from 13.5 to 49% (Kholeif and Fayez, 2008; Omar et al., 2008). Internationally, the *SENV* percentage was around 21% and reaching up to 69% in many previous studies (Kojima et al., 2003; Schroter et al., 2003; Yoshida et al., 2002). For the control group, we found the percentage of *SENV* to be 15% (3/20). This is in the range reported by previous Egyptian studies where it was found to be 16 and 20% among the controls (Omar et al., 2008; Sayed et al., 2006; Mohamed et al., 2011).

Regarding the CHC group, we reported *SENV* H, *SENV* D and combined H/D in 38.5, 15.4 and 11.5% respectively. This is comparable to the results of a previous Egyptian study, where the percentages were 42% (18/43), 16% (7/43) and 9% (4/43) respectively (Kholeif and Fayez, 2008). In agreement, many studies reported *SENV-H* to be more prevalent than *SENV-D* (Tezcan et al., 2009; Sayed et al., 2006; Loutfy et al., 2009). In Turkey, *SENV-H* DNA was found to be positive in 23.3% (7/30) of patients with CHC (Cakaloglu et al., 2008). On the other hand, *SENV* D was more prevalent in CHC patients (8.1%, 6/74) compared to only 5.4% (4/74) *SENV* H (Omar et al., 2008).

Concerning the CHB group, we reported *SENV* in 33.3% with *SENV* H again being the predominant genotype (33.3%) compared to only 16.7% for *SENV* D. Very limited Egyptian studies were found regarding

SENV in CHB patients. In Turkey, *SENV-H* DNA was found to be positive in 33.3% (10/30) of patients with CHB (Cakaloglu et al., 2008).

A recent study in Iran reported *SENV* in 59.3% of patients with HBV infection and in 73.5% of patients with HCV infection. *SENV-H* genotype was found to be positive in 31.39% (54/172) and 33.82% (23/68), and *SENV-D* genotype was detected in 27.91% (48/172) and 39.7% (27/68) of patients with CHB andCHC respectively (Dehkordi and Doosti, 2011).

The differences in the percentages of *SENV* detection in different countries and even in different regions in Egypt are accepted (Kholeif and Fayez, 2008; Omar et al., 2008). Similarly, in China, the prevalence varied significantly from one area to another (Tang et al., 2008). The frequency of *SENV* may vary demographically and geographically. The explanations for these differences are unknown, but they may result from interactions among behavioral, social, and biological factors (Bluthenthal et al., 1999). In addition, the difference in the rate of detection of *SENV* DNA in various studies may be due to differences in the quantity of *SENV* DNA in the sera, differences in the PCR primers used, or differences in the sensitivities of the assay systems used (Yoshida et al., 2002).

In the present study, we found that 34.6% of *SENV* (9/26) detected in CHC patients and 33.3% of *SENV* DNA (3/6) detected in CHB were not of H/D genotypes. These figures are higher than those reported in a previous study in the same hospital where *SENV* of non H/D genotype was detected in 14.3% of polytransfused patients (Mohamed et al., 2011).

Concerning the risk factors for *SENV*, we did not report any significant association with age, gender, duration of liver disease or history of blood transfusion. However, a previous study conducted on polytransfused patients in the same hospital, showed a significant difference between *SENV* positive and negative patients regarding the number of blood transfusions (Mohamed et al. 2011). This may be due to the limited number of patients giving a history of blood transfusion in this study (8/112). In addition, many studies reported that *SENV* was not associated with blood transfusion history (Yoshida et al., 2002; Tang et al., 2008) indicating that blood transfusion transmission is not the only way for people to be infected with *SENV* (Tang et al., 2008; Karimi-Rastehkenari and Bouzari, 2010). In agreement with our results, another Egyptian study reported a statistically insignificant difference between *SENV* positive and *SENV* negative liver patients regarding age and sex (Kholeif and Fayez, 2008). In Turkey, Cakaloglu et al also found no significant difference in the clinical features between *SENV-H*-positive and -negative patients with chronic viral hepatitis (Cakaloglu et al., 2008).

In the current study, we reported many important findings suggesting the absence of deleterious effects of *SENV* on the progression of chronic liver disease or even a possible protective role of *SENV* infection in patients with CHC. We demonstrated that *SENV* was negative in 90.9% of patients with CHC compared to 9.1% *SENV* positive among such group giving a statistically significant difference. In addition, we found a significantly higher percentage of patients showing twofold or more increase in the AST and ALT among *SENV* negative CHC patients (without cirrhosis) compared to *SENV* positive patients (P=0.04 and 0.03, respectively). Even for cirrhotic patients, the levels of ALP and serum total bilirubin were significantly higher in *SENV* negative patients compared to *SENV* positive patients (P values=0.01). A similar conclusion was reported in a previous Egyptian study where the prevalence of *SENV* infection among patients who have recovered from HCV infection was 61% (11/18) compared to 50% in HCV viremic patients (Loutfy et al., 2009). This finding was also observed in another study that reported that HCV was less prevalent among patients with *SENV-H* viremia (14%) than among patients without *SENV-H* viremia (34%) in an area of high HCV endemicity (Umemura et al., 2001). They also suggested a possible protective role of *SENV* against HCV or assistance with HCV clearance by some sort of virus to virus interaction, making *SENV* worthy of further studies on larger scales. For our patients with CHB, the effect of *SENV* was not conclusive; this may be due to the small number of patients included in the study.

Apart from the previously mentioned parameters, there were no statistically significant differences between *SENV*-positive and *SENV* negative chronic hepatitis patients regarding the demographic data and other laboratory findings. This is in agreement with many studies. A previous Egyptian study reported a statistically insignificant difference between CHC patients and HCV related HCC patients regarding *SENV* viraemia. They also reported no statistically significant difference regarding ALT, serum bilirubin, serum albumin and prothrombin time (Kholeif and Fayez, 2008). The same finding regarding the biochemical parameters were also reported by Alam El-Din et al. (2007). In addition, Yoshida et al. (2002) reported no significant differences between *SENV*-positive and *SENV* negative patients regarding serum albumin, total bilirubin and transaminase levels. Also, many studies reported absence of significant difference in the blood biochemical parameters between the *SENV* DNA-positive and -negative chronic hepatitis patients (Tangkijvanich et al., 2003; Moriyama et al., 2005).

Among our patients, we reported no histopathological differences and no liver cirrhosis related complications including HCC between *SENV* positive and negative chronic hepatitis patients. In concordance, Tangkijvanich et al. (2003) also reported no differences between *SENV*-infected and non-infected patients regarding severity of chronic liver disease and HCC. Recently, no evidence has been produced to indicate that SEN virus causes HCC (Kew, 2013). On the contrary, another study documented that *SENV* co-infection may influence the histopathological features of the livers of patients with

CHC but does not affect the outcome of patients with type C chronic liver disease. The histological features of the livers of *SENV* DNA-positive patients included more severe parenchymal inflammatory cell infiltration and more immune response (Moriyama et al., 2005).

The exact role of this virus in the pathogenesis of chronic liver diseases is not yet confirmed. Several studies reported that *SENV* does not seem to contribute to the pathogenesis of liver disease or worsens the course of coexistent liver disease or lead to the development of HCC from chronic liver disease (Yoshida et al., 2002; Akiba et al., 2005). Others suggested a protective role of *SENV* against HCV (Umemura et al., 2001; Loutfy et al., 2009). On the other hand, a recent Indian study reported that *SENV* appeared to cause liver damage in patients with hepatitis, but the number of hepatitis patients coinfected with *SENV* were very limited in that study (5 HBV and 3HCV) (Rizvi et al., 2013). So, further studies are needed to ascertain the association of *SENV* with liver disease.

From the present study we conclude that *SENV* virus does not worsen the progression of chronic viral hepatitis. A possible protective effect of *SENV* in CHC patients was reflected by changes in liver enzymes without histo-pathological changes which needs to be emphasized by further studies on larger scales

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

Akiba J, Umemura T, Alter HJ, Kojiro M, Tabor E (2005). SEN virus: epidemiology and characteristics of a transfusion-transmitted virus. Transfusion 45:1084-1092.

Alam El-Din HM, Abo-Shadi MA, Helal A (2007). Impact of SEN virus on immunoblot INNO-LIA HCV reactivity pattern and disease outcome in Egyptian hepatocellular carcinoma patients. Egypt. J. Immunol. 14(1):1-10.

Anwar W, Sarwar M, Hussain AB, Tariq WU, Saif M (2006). Significance of occult HBV infection in patients with chronic hepatitis C. J. Coll. Physicians Surg. Pak. 16(3):192-197.

Awadalla HI, Ragab MH, Osman MA, Nassar NA (2011). Risk Factors of Viral Hepatitis B among Egyptian Blood Donors. Br. J. Med. Med. Res. 1(1):7-13.

Biagini P (2009). Classification of TTV and related viruses (anelloviruses). Curr. Top. Microbiol. Immunol. 331:21-33.

Bluthenthal RN, Kral AH, Erringer EA, Edlin BR (1999). Drug paraphernalia laws and injection-related infectious disease risk among drug injectors. J Drug Issues 29:1-16.

Cakaloglu Y, Akyuz F, Bozaci M, Ibirisim D, Pinarbasi B, Demir K, Kaymakoglu S, Besisik F, Badur S, Okten A (2008). Prevalence and clinical significance of SEN-H virus in chronic hepatitis B, C and delta infections in Turkey. Turk. J. Gastroenterol. 19(2):104-108

Child CG, Turcotte JG (1964). Surgery and portal hypertension. In: The liver and portal hypertension. Edited by CG Child. Philadelphia: Saunders. pp. 50-64.

Dehkordi PG, Doosti A (2011). The prevalence of *SEN virus* infection in blood donors and chronic hepatitis B and C patients in Chaharmahal Va Bakhtiari province. J. Cell Anim. Biol. 5(8):182-186.

Huntzinger A (2009). AASLD Updates Chronic Hepatitis B Recommendations. Am. Fam. Physician 79(4):338-343.

Ishak K, Baptista A, Bianchi L, Callea F, Groote JD, Gudet F, Denk H, Desmet V, Korb G, MacSween RNM, Phillips MJ , Portmann BG, Poulsen H, Scheuer P J, Schmid M, Martin Schmid, Thaler H (1995). Histological grading and staging of chronic hepatitis. J. Hepatol. 22(6):696-699.

Jardi R, Rodrigez F, Buti M, Costa X, Cotrina M, Galimany R (2001). Role of hepatitis B, C and D viruses in dual and triple infections: influence of viral genotypes and hepatitis B precore and basal core promoter mutations on viral replicative interference. Hepatology 34:404-410.

Karimi-Rastehkenari A, Bouzari M (2010). High frequency of SEN virus viraemia in patients with hepatitis B virus and hepatitis C virus infection in Iran. Twentieth European Congress of Clinical Microbiology and Infectious Disease (ECCMID), Vienna, Austria, 10-13 April.

Kew MC (2013). Hepatitis viruses (other than hepatitis B and C viruses) as causes of hepatocellular carcinoma: an update. J. Viral Hepat. 20(3):149-157.

Kholeif L, Fayez D (2008). SEN Virus Detection in Egyptian Patients with Chronic Hepatitis C and Hepatitis C Related Hepatocellular Carcinoma. Egypt. J. Med. Microbiol. 17(1):91-97

Kojima H, Kaita KD, Zhang M, Giulivi A, Minuk GY (2003). Genomic analysis of a recently identified virus (SEN virus) and genotypes D and H by polymerase chain reaction. Antivir. Res. 60:27-33.

Loutfy SA, Hafez MM, Massoud WA, Fotuh NA, Moneer MM, Zaghloul HS (2009). SEN virus infection in Egyptian patients undergoing maintenance hemodialysis: prevalence and clinical importance. J. Microbiol. Immunol. Infect. 42:464-470

Mohamed IS, Thabit AG, Abd-El Rahman SA, Abdelmohsen E M, Seif Eldin S S, Ghandour A M (2011). Prevalence of SEN Virus Infection in Multitransfused Patients in Assiut University Hospitals, Egypt. J. Am. Sci. 7(1):687-696.

Mohamoud YA, Mumtaz GR, Riome S, Miller DW and Abu-Raddad LJ (2013). The epidemiology of hepatitis C virus in Egypt: a systematic review and data synthesis. BMC Infect. Dis. 13:288.

Moriyama M, Mikuni M, Matsumura H, Nakamura H, Oshiro S, Aoki H, Shimizu T, Yamagami H, Shioda A, Kaneko M, Tanaka N,Arakawa Y (2005). SEN virus infection influences the pathological findings in liver but does not affect the incidence of hepatocellular carcinoma in patients with chronic hepatitis C and liver cirrhosis. Liver Int. 25(2):226-235.

Mu SJ, Du J, Zhan LS, Wang HP, Chen R, Wang QL, Zhao WM (2004). Prevalence of a newly identified *SEN virus* in China. World J. Gastroenterol. 10(16):2402-2405.

Omar M, El-Din SS, Fam N, Diab M, Shemis M, Raafat M, Seyam M, Hssan M, Badawy A, Akl M, Saber M (2008). *SEN virus* infection in Egyptian patients with chronic hepatitis C and patients undergoing hemodialysis. Medscape J. Med. 10(12):290.

Pirovano S, Bellinzoni M. Ballernin C, Marini M, Albertini A, and Imberti L (2002). Transmission of *SENV* infection from mothers to their babies. J. Med. Virol. 66:421-26.

Pugh RNH, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R (1973). Transection of the esophagus in bleeding oesophageal varices . Br. J. Surg. 60:648-652.

Rizvi M, Jahan S, Azam M, Ajmal MR, Shukla I, Malik A, Sultan A (2013). Prevalence and assessment of role of SEN virus in acute and chronic hepatitis in India. Trop. Gastroenterol. 34(4):227–234

Sagir A, Kirschberg O, HeintgesT, Erhardt A, Haussinger D (1994). SEN virus infection. Rev. Med. Virol. 14:141-48.

Sayed NM, Darwish NM, Abdel Aziz AA and Abdel Aziz AA (2006). Detection of SEV virus DNA among intra venous drug users (IVDUs) and blood donors. Egypt. J. Gastroenterol. (14)2:101-112.

Schreter I, Kristian P, Jarcuska P, Porubcin S, Siegfried L,Birosova E (2006). Detection of SEN virus in the general population and different risk groups in Slovakia. Folia Microbiol. 51:223-231.

Schroter M, Laufs R, Zollner B, Knodler B, Schafer P, Feucht HH (2003). A novel DNA virus (SEN) among patients on maintenance hemodialysis: prevalence and clinical importance. J. Clin. Virol. 27:69-73.

Sugiura T, Goto K, Imamine H, Ando T, Ban K, Sugiyama K, Togari H (2004). Prevalence of SEN virus among children in Japan. Virus Res.

100:223-228.
Tahan V, Ozdogan O, Tozun N (2003). Epidemiology of viral hepatitis in the Mediterranean basin. Rocz. Akad. Med. Bialymst 48:7-11.
Tang Z-H, Chen X-H, Yu Y-S, Zang G-Q (2008). Prevalence and clinical significance of SEN virus infection in patients with non A-E hepatitis and volunteer blood donors in Shanghai. World J. Gastroenterol. 14(26):4204-4208
Tangkijvanich P, Theamboonlers A, Sriponthong M, Thong-Ngam D, Kullavanijaya P, Poovorawan O (2003). SEN virus infection in patients with chronic liver disease and hepatocellular carcinoma in Thailand. J. Gastroentrol. 38(2):142-48.
Tezcan S, Delialioglu N, Serin MS, Aslan G, Tiftik N, Emekdas G (2009). Prevalence of SEN virus genotype-D and genotype-H among haemodialysed patients. Turk. J. Med. Sci. 39(3):397-403
Umemura T, Alter HJ, Tanaka E, Orii K, Yeo AET, Shih JW-K, Matsumoto A, Yoshizawa K, Kiyosawa K (2002). SEN virus: response to interferon alpha and influence on the severity and treatment response of coexistent hepatitis C. Hepatology 35:953-962.
Umemura T, Alter HJ, Tanaka E, Yeo AE, Shih JW, Orii K, Matsumoto A, Yoshizawa K, Kiyosawa K (2001). Association between SEN virus infection and hepatitis C in Japan. J. Infect. Dis. 184:1246-1251.
Wang LY, Ho TY, Chen MC, Yi CS, Hu CT, Lin HH (2007). Prevalence and determinants of *SENV* viremia among adolescents in an endemic area of chronic liver diseases. J. Gastroenterol. Hepatol. 22: 171-176.
Yahia M (2011). Global health: A uniquely Egyptian epidemic. Nature 474(7350 Suppl.). S12-3. World Journal of Gastroente WHO: Hepatitis B surface Ag assays; operational characteristics. Phase 1.Report, World Health Organization, 2004, (WHO/BCT/BTS/01.4). Yoshida H, Kato n, Shiratori Y, Shao R, Wang Y, Shiina S, and Omata M (2002). Weak Association between SEN Virus Viremia and Liver Disease. J. Clin. Microbiol. 40(9):3140-3145.

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Full Length Research Paper

Additive antibacterial activity of naringenin and antibiotic combinations against multidrug resistant *Staphylococcus aureus*

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Methicillin-resistant *Staphylococcus aureus* has been causing numerous problems in the health care sector. This is mainly due to its ability to develop resistance to a number of antibiotics used to treat staphylococcal infections. Medicinal plants have been used to treat various ailments over the years and are generating a lot of interest as alternative treatment options. Naringenin is a plant derived flavonoid that possesses antibacterial properties, among others. This study assessed the effect of combinations of naringenin and four antibiotics against two *Staphylococcus aureus* strains. The minimum inhibitory concentrations were determined using the disk diffusion and broth microdilution assays. In the disk diffusion assay, naringenin did not inhibit bacterial growth, nor did it enhance the antibacterial activity of the antibiotics in the combination study. This was attributed to its slow rate of diffusion out of the disks. On the contrary, in the broth microdilution assay, naringenin exhibited additive effects when combined with the antibiotics (at sub-inhibitory concentrations). These results show the potential of naringenin as an antibacterial agent. Furthermore, the additive effects observed at low naringenin concentrations showed that it can potentially be used in combination with antibiotics against multidrug resistant bacteria.

Key word: Staphylococcus aureus, MSSA, MRSA, antibiotics, flavonoids, disk diffusion assay, broth dilution assay, MIC.

INTRODUCTION

Premature deaths from infectious diseases are a global problem (Ahmad and Beg, 2001). Moreover, as a result of the widespread overuse of antimicrobial agents to treat these infectious diseases, bacteria have developed ways to minimise the effects of antibiotics through evolutionary adaptations, leading to the emergence of antibiotic resistant pathogens (Fielding et al., 2012). *Staphylococcus aureus* (*S. aureus*) is the leading cause of both nosocomial and community acquired infections (Lowy, 1998) and is often isolated from the bloodstream, skin and soft tissue infections (Bernards et al., 1998; Pfaller et al., 1999; Deresinski, 2005). Disconcertingly,

because methicillin-resistant *S. aureus* (MRSA) strains carry multiple genes of antibiotic resistance, many have developed resistance to a variety of commonly used antimicrobials and may, in future, become resistant to newly developed antimicrobial agents as well (Vijaya et al., 2013; Xia et al., 2013; Medeiros et al., 2014; Szweda et al., 2014). Hence, alternative methods, such as natural plant products, are being studied for their potential to alleviate the burden created by the multidrug resistant bacteria such as MRSA.

Flavonoids are plant-derived compounds reported to possess numerous therapeutic properties which include direct antibacterial activity, as well as synergistic activity when used in combination with antibiotics (Cushnie and Lamb, 2005). Historically, flavonoids are used to treat and prevent various infectious and toxin-mediated diseases, including sores, wound infections (Cushnie and Lamb, 2005), acne, respiratory infections (Gutierrez et al., 2008), gastrointestinal disease (Shan et al., 2007) and urinary tract infections (Ngueyem et al., 2009), among others. For this reason, flavonoids have been extensively studied for their antibacterial properties (Cushnie et al., 2003; Cushnie and Lamb, 2005; Mehndiratta et al., 2010; Cushnie and Lamb, 2011). Naringenin is a flavanone, a type of flavonoid, found in grapefruit and tomatoes. It contains two benzene rings linked together with a heterocyclic pyrone ring (a class of cyclic compounds) (Tripoli et al., 2007). It is believed to possess antibacterial (Celiz et al., 2011), antioxidant, anticancer anti-inflammatory and immunomodulatory properties (Goldwasser et al., 2011; Khachatoorian et al., 2012; Lee et al., 2013). The purpose of this study was to evaluate the antibacterial activities of naringenin on its own, as well as assess the combinatory antibacterial effects of naringenin and antibiotics against methicillin-sensitive and methicillin-resistant strains of *S. aureus* (*i.e.* MSSA and MRSA, respectively).

MATERIALS AND METHODS

Naringenin and the antibiotics (ampicillin, methicillin, tetracycline and vancomycin) used in this study were purchased from Sigma-Aldrich (USA). *S. aureus* ATCC 25923 (MSSA) and ATCC 33591 (MRSA) were obtained from the American Type Culture Collection (ATCC). Antibacterial activity and determination of minimum inhibitory activity was determined as per the Clinical and Laboratory Standards Institute (CLSI) guidelines.

In the disk diffusion assay, naringenin was made to yield twofold serial dilutions (0.002 to 2 $mg.ml^{-1}$); the antibiotic concentrations used were 1.25, 2.5 5, 12, 25 and 50 $\mu g.ml^{-1}$. Whereas the naringenin concentrations were made up in dimethyl sulfoxide (DMSO), the antibiotic dilutions were made up in distilled water. 9 mm sterile filter disks (Lasec) were impregnated with 40 µl of naringenin or antibiotic and allowed to dry at 37°C; this was done in triplicate. DMSO control disks were also included to assess its effect on bacterial growth. Bacterial suspensions were prepared by culturing an inoculum of each strain in 5 ml Tryptone soy broth (TSB) and incubated overnight at 37°C. Next, 100 µl of each overnight suspension was re-cultured in 5 ml fresh broth and incubated for 1-2 h at 37ºC and then adjusted to a bacterial density of 1×10^8 to 2×10^8 $CFU.ml^{-1}$. 100 µl of each suspension was spread onto Tryptone soy agar (TSA) plates. The disks impregnated with naringenin or antibiotics, as well as DMSO, were placed on the plates and incubated at 37ºC for 18-24 h. For the combination studies, the antibiotic concentrations chosen were; 1.25, 2.5, 5, and 12 $\mu g.ml^{-1}$ for tetracycline, ampicillin, methicillin and vancomycin, respectively. These concentrations were chosen because they produced zones that were big enough to show antagonism but also small enough to show synergy. These antibiotic concentrations were individually combined with the naringenin concentrations (0.002 to 2 $mg.ml^{-1}$) and loaded onto disks as described above. Zones of inhibition were measured and recorded.

The broth microdilution assay was carried out in 96 well microtitre plates. Twofold serial dilutions of the antibiotics were made to yield concentrations ranging from 0.0156 to 1 $\mu g.ml^{-1}$ for ampicillin, 0.0313 to 16 $\mu g.ml^{-1}$ for methicillin, 0.0625 to 32 $\mu g.ml^{-1}$ for tetracycline and 0.0039 to 5 $\mu g.ml^{-1}$ for vancomycin, which were made up in distilled water. Naringenin concentrations remained the same as those used in the disk diffusion assay. The solvent of choice for naringenin, in the microdilution assay, was methanol. The other solvents, such as DMSO, caused naringenin to precipitate when added to the wells while methanol did not. The naringenin experimental wells contained 10 µl naringenin and 90 µl of TSB (this was done to ensure that the final methanol concentration in the wells was at 5%), the antibiotic experimental wells contained 20 µl antibiotic and 80 µl TSB. 100 µl of either MSSA or MRSA culture was added to obtain a final volume of 200 µl. Control wells containing a final volume of 5% methanol, distilled water, bacteria alone and TSB alone were also included and the experiments were done in triplicate. The plates were then sealed and incubated at 37ºC with continuous shaking for 18 h to allow detection of viability. The naringenin and antibiotic experimental plates were then read at 620 nm in a plate reader. The MIC was determined as the lowest concentration that inhibited bacterial growth. For the combinations studies, the antibiotic concentrations chosen were 0.0625 $\mu g.ml^{-1}$ for both ampicillin and methicillin, 0.25 $\mu g.ml^{-1}$ for tetracycline and 0.0039 $\mu g.ml^{-1}$ for vancomycin. These concentrations were selected because they did not have any inhibitory activity against the bacterial strains (results not shown). Naringenin was serially diluted in TSB (0.002 to 2 $mg.ml^{-1}$) and 20 µl of each antibiotics were added to the wells followed by 100 µl of the two bacterial strains as outlined earlier.

Mathematical synergistic ratios (SR) for the combination of naringenin and antibiotics were calculated using the Abbott formula $\%C_{exp}=A+B-(AB/100)$, where A was the control level of the antibiotic, B the control level of naringenin and C_{exp} represented the expected efficacy of the mixture (naringenin and antibiotic combination) (Levy et al., 1986). After calculating the $\%C_{exp}$ (expected efficacy), the SR was obtained using the formula $SR=C_{obs}/C_{exp}$, with C_{obs} representing the observed efficacy. The ratios obtained after these calculations enabled determination of

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Table 1. Resistance (R) and susceptibility (S) of *S. aureus* strains to antibiotics in the disk diffusion assay.

Antibiotic		MIC (µg.ml^{-1}) / Zone of inhibition (mm)			
		MSSA ATCC 25923		MRSA ATCC 33591	
Ampicillin	R	2.5	19±0.9	50	12±0
	S	5	31±1.2	-	-
Methicillin	R	1.25	-	50	-
	S	5	19±0.9	-	-
Tetracycline	R	5	11±0	50	10±0
	S	12	21±0.5	-	-
Vancomycin	R	-	-	-	-
	S	12	15±0.9	12	15±0.2

CLSI breakpoints for susceptibility and resistance criteria for zone diameter; ampicillin (10 µg) ≤28=R, ≥29=S; methicillin (5 µg) ≤ 9 = R, 10-13 = I, ≥ 14 = S; tetracycline (30 µg) ≤ 14 = R, 15-18 = I, ≥ 19 = S; vancomycin (30 µg) ≥ 15 =S (no breakpoints for R or I for vancomycin). R = resistant; I = intermediate; S = susceptible. Zones of inhibition expressed as mean of three replicates ± standard deviation (SD).

synergistic interaction in the mixture. If SR is greater than 1.5, then synergistic interactions are present; if between 0.5-1.5 then the interaction is additive; and if the SR is below 0.5, then the effect is antagonistic (Gisi and Binder et al., 1985; Gisi, 1996). This enabled the evaluation of the level of interaction between naringenin and antibiotics.

RESULTS AND DISCUSSION

In the disk diffusion assay, naringenin did not show any inhibitory activity against the *S. aureus* strains, neither did naringenin enhance the antibacterial activities of the antibiotics. However, lack of inhibition zones does not always mean the compound does not possess antibacterial activities. It could be attributed to its slow diffusion ability (Moreno et al., 2006). The various antibiotic concentrations exhibited some antibacterial activity against the MSSA ATCC 25923 strain. The MRSA ATCC 33591 strain was only susceptible to vancomycin with it being resistant even at concentrations of 50 µg.ml^{-1} for the other antibiotics (Table 1). The growth of MSSA was inhibited by 5 µg.ml^{-1} ampicillin, 5 µg.ml^{-1} methicillin and 12 µg.ml tetracycline though inhibitory activity was lost at 5 µg.ml^{-1}. MSSA ATCC 25923 and MRSA ATCC 33591 were both inhibited by a vancomycin concentration of 12 µg.ml^{-1} (as seen by the inhibition zones of ≥ 15 mm, as outlined by the CLSI breakpoints for sensitivity). The MIC breakpoints for resistance and susceptibility were determined according to those outlined by the CLSI (CLSI, 2007).

Although DMSO has some antibacterial activity (Basri and Zin, 2008), it did not inhibit the growth of the bacterial strains when used as control. Previous studies show that flavonoids diffuse slowly out of disks, thereby affecting their antibacterial activities (Zheng et al., 1996). This could explain why naringenin did not have any inhibitory effect or enhance the antibacterial effect of the antibiotics in the disk diffusion assay.

However, in the broth dilution assay, naringenin enhanced the antibacterial activities of the four antibiotics at concentrations that were sub-inhibitory as seen by the synergistic ratios (SR) obtained in Table 2. This was despite naringenin showing only minimal antibacterial effect on its own against *S. aureus*. The results obtained in this study showed that naringenin had an additive effect on the antibacterial activity of the antibiotics (as determined by the calculated SR of between 0.66 and 0.95 for the different combinations. These values depict the lowest and highest SR obtained). Studies have shown that naringenin has an additive effect when combined with vancomycin and oxacillin against vancomycin-intermediate *S. aureus* (VISA) (Bakar et al., 2012). In the naringenin-antibiotic combinations, an increase in antibiotic antibacterial activity was seen at naringenin concentrations of; 0.063 to 0.25 mg.ml^{-1} for ampicillin, 0.25 to 1 mg.ml^{-1} for methicillin, 0.125 to 5 mg.ml^{-1} for tetracycline and 0.016 to 1 mg.ml^{-1} for vancomycin (with the greatest effect observed at 0.016, 0.063 and 0.125 mg.ml^{-1}) for the MSSA strain. With the MRSA strain, an increase in antibiotic antibacterial activity was seen at naringenin concentrations of; 0.25 mg.ml^{-1} for ampicillin, 0.25 to 1 mg.ml^{-1} for methicillin, 0.25 to 0.5 mg.ml^{-1} for tetracycline and 0.125 to 0.25 mg.ml^{-1} for vancomycin. The increased antibacterial effect

Table 2. Antibacterial activity of naringenin in combination with antibiotics against methicillin-sensitive (MSSA) and –resistant (MRSA) *S. aureus*

Drugs (µg.ml^{-1})	Naringenin (mg.ml^{-1}) + antibiotic combinations						
MSSA ATCC 25923							
Ampicillin (0.0625) +	0.016	0.031	0.063	0.125	0.25	0.5	1
Bacterial growth (%)	86	82	**72**	**69**	**73**	83	82
SR	0.87	0.83	0.73	0.67	0.74	0.84	0.83
Methicillin (0.0625) +	0.016	0.031	0.063	0.125	0.25	0.5	1
Bacterial growth (%)	92	93	92	90	**84**	**82**	**82**
SR	0.93	0.94	0.93	0.91	0.85	0.83	0.83
Tetracycline (0.25)	0.016	0.031	0.063	0.125	0.25	0.5	1
Bacterial growth (%)	92	92	91	**88**	**84**	**84**	90
SR	0.93	0.93	0.92	0.89	0.85	0.85	0.91
Vancomycin (0.0039)	0.019	0.031	0.063	0.125	0.25	0.5	1
Bacterial growth (%)	**66**	82	**70**	**71**	77	78	85
SR	0.66	0.83	0.70	0.72	0.78	0.80	0.86
MRSA ATCC 33591							
Ampicillin (0.0625) +	0.016	0.031	0.063	0.125	0.25	0.5	1
Bacterial growth (%)	95	95	95	93	**89**	91	92
SR	0.96	0.96	0.96	0.94	0.90	0.92	0.93
Methicillin (0.0625)	0.016	0.031	0.063	0.125	0.25	0.5	1
Bacterial growth (%)	93	93	94	92	**88**	**89**	**89**
SR	0.93	0.93	0.94	0.92	0.89	0.90	0.90
Tetracycline (0.25)	0.016	0.031	0.063	0.125	0.25	0.5	1
Bacterial growth (%)	95	95	94	94	**89**	**87**	93
SR	0.95	0.95	0.94	0.94	0.89	0.87	0.94
Vancomycin (0.0039)	0.016	0.031	0.063	0.125	0.25	0.5	1
Bacterial growth (%)	95	94	**88**	**77**	**81**	84	84
SR	0.95	0.94	0.88	0.77	0.81	0.85	0.85

The numbers in bold represent points at which the greatest reduction in bacterial % growth was observed. SR = synergistic ratio. Naringenin exhibited an additive effect when combined with the antibiotics.

was accompanied by a decrease in bacterial growth at the concentrations outlined above (Table 2). Studies have shown that naringenin, as well as its derivatives, exhibit increased antibacterial activities. In fact, data demonstrate that an increase in chain length (10-12 carbon atoms) and modifying its structure increased the antibacterial activities. This shows that the structure of naringenin is important for interaction with the antibiotics in combination studies (Celiz et al., 2011; Lee et al., 2013).

Synergy studies of natural products and antibiotics are increasing in order to assess their combination effects (Olajuyigbe and Afolayan, 2013). Studies of natural plant products such as *Salvia afficinalis* (*S. afficinalis*) oils, reveal that they effectively inhibited the growth of *S. aureus* and *Streptococcus* group D at a 20 µl/ml concentration and show enhanced antibacterial activity when compared to most known antibiotics in multidrug resistant bacteria (Khalil and Li, 2010). Extracts of *Rehum palmatum*, *Cassia angustifolia* and *Glycyrrhiza glabra* also exhibited antibacterial activity against antibiotic-resistant bacteria. These extracts enhanced the antibacterial activities of the antibiotics used hence showing their synergistic effect (Dawoud et al., 2013). Liu and colleagues showed that despite numerous *S. aureus* clinical isolates being resistant to fluroquinolones, they can be combined with the flavonoid biochanin A (BCA) creating a potent antimicrobial agent (Liu et al., 2011). Analysis of the ethanol bark extract of *Ziziphus mucronata* in combination with antibiotics against clinically important bacteria revealed both additive and synergistic effects. These results further agree with other published data stating that natural plants and plant products enhance antibacterial activities of antibiotics.

The data generated in this study demonstrated the flavonoids possess the ability of enhancing the antibacterial activities of antibiotics to which bacteria are resistant. However, further studies explaining the precise mode of action of flavonoids, including other plant extracts, against multidrug resistant pathogens will be

beneficial in their overall treatment. Despite the availability of various methods for evaluating the MIC of plants extracts or plant compounds, not all produce similar results (Jorgensen and Ferraro, 2009). For instance, the disk diffusion assay does not allow for natural antimicrobial compounds that are barely soluble or insoluble in water to diffuse uniformly from the disks. Consequently, these compounds also do not diffuse uniformly through agar media when the agar diffusion assay is used (Mann and Markham, 1998). A study evaluating the disk diffusion and broth microdilution assays showed that the broth microdilution assay was a better technique for assessing the antibacterial activity of plant extracts or plant compounds (Klancnik et al., 2010). Another study showed that results obtained with the broth microdilution assay were more reproducible (99%) when compared to the macrodilution technique (89%) (Murray and Hospenthal, 2004). This study further showed that the MICs obtained in the microdilution assay were lower than those obtained in the macrodilution assay (Murray and Hospenthal, 2004). Another advantage of the broth microdilution assay is that it produces quantitative results and also allows for the compounds to interact with the bacterial strains in suspension. This could explain why the MICs generated in the broth microdilution assay were lower than those in the disk diffusion assay.

In conclusion, the burden created by multidrug resistant bacteria, which is worsened by the lack of new therapeutic drugs, is mainly brought about by the duration it takes for new therapeutic agents to be tested and released on the market. It is for this reason that alternative treatment options, such as plants and plant products, are generating a tremendous amount of interest. Antimicrobial combinations are currently used in medicine to try and limit the rate of antibiotic-resistance and should be encouraged. This study showed that naringenin displayed additive effects when combined with the different antibiotics at sub-inhibitory concentrations. These results further showed that naringenin has the capacity to be used as a therapeutic agent, or to enhance the antibacterial effects of various antibiotics. Further research to determine its exact mechanism of action would be an added advantage in understanding how naringenin interacts with these antibiotics.

Conflict of interests

The authors did not declare any conflict of interest.

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REFERENCES

Ahmad I, Beg AZ (2001). Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. J. Ethnopharmacol. 74(2):113-123.

Bakar NS, Zin NM, Basri DF (2012). Synergy of flavone with vancomycin and oxacillin against vancomycin-intermediate Staphyloccus aureus. Pak. J. Pharm. Sci. 25(3):633-638.

Basri DF, Zin NM (2008). Synergistic effects of phytochemicals and oxacillin on laboratory passage derived vancomycin intermediate Staphylococcus aureus strain. J. Med. Sci. 8(2):131-136.

Bernards AT, Frénay HM, Lim BT, Hendriks WD, Dijkshoorn L, van Boven CP (1998). Methicillin-resistant Staphylococcus aureus and Acinetobacter baumannii: an unexpected difference in epidemiologic behavior. Am. J. Infect. Control 26(6):544-551.

Celiz G, Daz M, Audisio MC (2011). Antibacterial activity of naringin derivatives against pathogenic strains. J. Appl. Microbiol. 111(3):731-738.

CLSI (2007). Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Information Supplement. CLSI CLSI M100-S17 [ISBN 1-56238-625-5]: 44-49 and 110-113.

Cushnie TP, Hamilton VE, Lamb AJ (2003). Assessment of the antibacterial activity of selected flavonoids and consideration of discrepancies between previous reports. Microbiol. Res. 158(4):281-289.

Cushnie TP, Lamb AJ (2005). Antimicrobial activity of flavonoids. Int. J. Antimicrob. Agents 26(5):343-356.

Cushnie TP, Lamb AJ (2011). Recent advances in understanding the antibacterial properties of flavonoids. Int. J. Antimicrob. Agents 38(2):99-107.

Dawoud MEA, Mawgoud YA, Gouda Dawoud TM (2013). Synergistic interactions between plant extracts, some antibiotics and/or their impact upon antibiotic-resistnt bacterial isolates. Afr. J. Biotechnol. 12(24):3835-3846.

Deresinski S (2005). Methicillin-resistant Staphylococcus aureus: an evolutionary, epidemiologic, and therapeutic odyssey. Clin. Infect. Dis. 40(4): 562-573.

Fielding BC, Mnabisa A, Gouws PA, Morris T (2012). Antimicrobial-resistant Klebsiella species isolated from free-range chicken samples in an informal settlement. Arch. Med. Sci. 8(1):39-42.

Gisi U (1996). Synergistic Interaction of Fungicides in Mixtures. Am. Phytopathol. Soc. 86(11): 1273-1274.

Gisi U, Binder H, Rimbach E (1985). Synergistic interactions of fungicides with different modes of action. Trans. Br. Mycol. Soc. 85:299-306.

Goldwasser J, Cohen PY, Lin W, Kitsberg D, Balaguer P, Polyak SJ, Chung RT, Yarmush ML, Nahmias Y (2011).Naringenin inhibits the assembly and long-term production of infectious hepatitis C virus particles through a PPAR-mediated mechanism. J. Hepatol. 55(5):963-971.

Gutiérrez RM, Mitchell S, Solis RV (2008). Psidium guajava: a review of its traditional uses, phytochemistry and pharmacology. J. Ethnopharmacol. 117(1):1-27.

Jorgensen JJ, Ferraro MJ (2009). Antimincrobial Susceptibility Tesing: A Review of General Principles and Contemporary Practices. Med. Microbiol. 49:1749-1755.

Khachatoorian R, Arumugaswami V, Raychaudhuri S, Yeh GK, Maloney EM, Wang J, Dasgupta A, French SW (2012). Divergent antiviral effects of bioflavonoids on the hepatitis C virus life cycle. Virology 433(2):346-355.

Khalil R, Li Z (2010). Antimicrobial activity of essential oil of Salvia afficinalis L. collected in Syria. Afr. J. Biotechnol. 10(42):8397-8402.

Klancnik A, Piskernik S, Jersek B, Mozina SS (2010). Evaluation of diffusion and dilution methods to determine the antibacterial activity of plant extracts. J. Microbiol. Methods 81(2): 121-126.

Lee KA, Moon SH, Lee JY, Kim KT, Park YS, Paik HD (2013). Antibacterial activity of a novel flavonoid, 7-O-Butyl Naringenin, against

methicillin-resistant *Staphylococcus aureus* (MRSA). Food Sci. Biotechnol. 22(6):1725-1728.
Levy Y, Benderly M, Cohen Y, Gisi U, Bassand D (1986). The joint action of fungicides in mixtures: comparison of two methods for synergy calculation. EPPO Bull. 16:651-657.
Liu G, Liang JC, Wang XL, Li ZH, Wang W, Guo N, Wu XP, Shen FG, Xing MX, Liu LH, Li L, Liu MY, Yu L (2011). *In vitro* synergy of biochanin A and ciprofloxacin against clinical isolates of *Staphylococcus aureus*. Molecules 16(8):6656-6666.
Lowy FD (1998). *Staphylococcus aureus* infections. N. Engl. J. Med. 339(8):520-532.
Mann CM, Markham JL (1998). A new method for determining the minimum inhibitory concentration of essential oils. J. Appl. Microbiol. 84(4):538-544.
Medeiros Barreto H, Cerqueira Fontinele F, Pereira de Oliveira A, Arcanjo DD, Cavalcanti Dos Santos BH et al. (2014). Phytochemical Prospection and Modulation of Antibiotic Activity *in vitro* by Lippia origanoides H.B.K. in methicillin resistant *Staphylococcus aureus*. Biomed. Res. Int. 305610:1-7.
Mehndiratta PL, Gur R, Saini S, Bhalla P (2010). *Staphylococcus aureus* phage types and their correlation to antibiotic resistance. Indian J. Pathol. Microbiol. 53(4):738-741.
Moreno S, Scheyer T, Romano CS, Vojnov AA (2006). Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. Free Radic. Res. 40(2):223-231.
Murray CK, Hospenthal DR (2004). Broth microdilution susceptibility testing for Leptospira spp. Antimicrob. Agents Chemother. 48(5):1548-1552.
Ngueyem TA, Brusotti G, Caccialanza G, Finzi PV (2009). The genus Bridelia: a phytochemical and ethnopharmacological review. J. Ethnopharmacol. 124(3):339-349.
Olajuyigbe OO, Afolayan AJ (2013). Evaluation of combination effects of ethanolic extract of *Ziziphus mucronata* Willd. subsp. mucronata Willd. and antibiotics against clinically important bacteria. Sci. World J. 769594:1-9.
Pfaller MA, Jones RN, Doern GV, Sader HS, Kugler KC, Beach ML (1999). Survey of blood stream infections attributable to gram-positive cocci: frequency of occurrence and antimicrobial susceptibility of isolates collected in 1997 in the United States, Canada, and Latin America from the Sentry Antimicrobial Surveillance Program. Sentry Participants Group. Diagn. Microbiol. Infect. Dis. 33(4):283-297.
Shan B, Cai YZ, Brooks JD, Corke H (2007). Antibacterial properties and major bioactive components of cinnamon stick (Cinnamomum burmannii): activity against foodborne pathogenic bacteria. J. Agric. Food Chem. 55(14):5484-5490.
Szweda P, Schielmann M, Frankowska A, Kot B, Zalewska M (2014). Antibiotic Resistance in Staphylococcus aureus Strains Isolated from Cows with Mastitis in Eastern Poland and Analysis of Susceptibility of Resistant Strains to Alternative Nonantibiotic Agents: Lysostaphin, Nisin and Polymyxin B. J. Vet. Med. Sci. 76(3):355-362.
Tripoli E, La Guardia M, Giammanco S, Di Majo D, Giammanco M (2007). Citrus flavonoids:molecular structure, biological activity and nutritional properties: a review. Food Chem. 104(2):466-479.
Vijaya Lakshmia A, Harasreeramulub S, Satyanarayana Raju DVV (2013). Assessment of antibacterial potential of selected medicinal plants and their interactions with antibiotics on MRSA in the health care workers of Visakhapatnam hospitals. J. Pharm. Res. 589-592.
Xia J, Gao J, Kokudo N, Hasegawa K, Tang W (2013). Methicillin-resistant Staphylococcus aureus antibiotic resistance and virulence. Biosci. Trends 7(3):113-121.
Zheng WF, Tan RX, Yang L, Liu ZL (1996). Two flavones from Artemisia giraldii and their antimicrobial activity. Planta Med. 62(2):160-162.

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Effect of rock phosphate enriched compost and chemical fertilizers on microbial biomass phosphorus and phosphorus fractions

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The aim of this research work was to prepare rock phosphate enriched compost using low-grade mineral such as rock phosphate (RP) mixed with rice straw and *Aspergillus awamori* and to study their effect on microbial biomass phosphorus (MBP), phosphatase activity and phosphorus (P) fractions in a wheat-soybean cropping system. The experiment was carried out in a randomized block design with four treatments namely, control, recommended dose of NPK fertilizers (100% RDF), rock phosphate enriched compost (RPEC) at the rate of (5 t ha^{-1} and 50% RDF + Rock phosphate enriched compost (RPEC) at 5 t ha^{-1}. Application of RPEC at 5 t ha^{-1} along with 50% recommended dose of chemical fertilizers (RDF) significantly improved microbial biomass P (MBP) (5.62 and 4.28 mg kg^{-1} soil) and alkaline phosphatase activity (194.0 and 174.0 µg PNP g^{-1} soil h^{-1}) in surface (0-15 cm) and sub-surface (15-30 cm) soil respectively, than unfertilized control plot after harvest of wheat. The magnitude of changes of P fractions as well as microbial activities was higher in surface soil than sub-surface soil. Data generated from the field study revealed that phosphorus (P) fractions significantly increased due to application of RPEC either alone or in combination of chemical fertilizers over unfertilized control plot. Application of RPEC plus chemical fertilizers significant increased Olsen P compared to unfertilized control plot after harvest of wheat and soybean. Treatment T_4 increased by 68.8 and 58.7% higher Olsen-P over control at 0-15 and 15-30 cm soil depth, respectively after wheat harvest.

Key words: Microbial biomass P, phosphatase enzyme, phosphorus fractions, Olsen P, rock phosphate, rice straw.

INTRODUCTION

Shortage of nutrients in soil particular to phosphorus (P) and high cost of chemical fertilizers in the present days has imposed an interest on recycling of agricultural and industrial waste for crop production; hence, research priorities have been directed toward finding alternative sources of P fertilizer. Over the past ten years, the global

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paddy rice output on an average was about 664.3 Mt (FAO 2013). However, the produced rice straw has limited use as an animal feed because of high silica content (Van Soest, 2006). Direct incorporation of the rice straw into soil is also limited as it may cause certain agronomic problems such as temporary immobilization of nutrients and associated crop yield reduction (Yadvinder-Singh et al., 2005). As a result, a large amount of produced straw is left as unutilized, which is mostly burnt on-farm, although burning of the straw in situ is the most discouraged option as it emits air pollutants and causes considerable carbon and nutrients loss.

The cost of phosphatic fertilizer is very high all over the India along the world. Presently, about 160 Million tonnes (Mt) of rock phosphate (RP) deposit is available in India (FAI, 2011). Unfortunately, most of them are designated as low-grade material containing less than 20% P_2O_5 and considered unsuitable for manufacturing of conventional commercial phosphatic fertilizers. Therefore, it is the need of the hour to find alternative indigenous sources of plant nutrients which could supply P for sustainable crop production, if not fully, but partially to reduce the reliance on costly chemical fertilizer. Attempts were made to recycling crop residues (rice straw) through composting with rock phosphate and bioinoculant which may serves as a supplement source of plant nutrients (Biswas and Narayanasamy, 2006).

The availability of P in rock phosphate thus could be mobilized through composting technology where unavailable P is expected to convert into plant available forms because of the acidic environment prevailing during composting, thereby improved the nutrient content of compost. Nishanth and Biswas, 2008). Moreover, farmyard manure (FYM) and traditional compost contain very small amount of nutrients, particularly P content and even difficult to obtain required quantity for substituting chemical fertilizers. Rock phosphate enriched compost prepared with rice straw and agro-based industrial wastes could be used as alternatives of FYM or traditional compost to sustain soil physical, chemical and biological quality.

The extent to which organic matter contributes to soil quality depend not only the quality of the organic matter but also on soil microbial activity and environmental conditions (Ouedraogo et al., 2001). Soil enzyme activities are very important component in agriculture for their role in the nutrient cycling and were considered to be early indicators of specific biochemical reactions in soil because of their relationship to soil biology and rapid response to changes in soil management (Bandick and Dick, 1999). Tarafdar and Jungk (1987) and Chen et al. (2002) suggested that higher phosphatase in the rhizosphere, compared to the bulk soil, can induce significant depletion of organic P forms in the rhizosphere. Phosphatase plays an important role in transforming organic phosphorus (P) into inorganic forms for plants, particularly when P availability limits plant productivity (Pan et al., 2013). Therefore, phosphatase activities have been regarded as an important factor for maintaining and controlling mineralization rate of soil organic P, and a good indicator of P-deficiency (Vance et al., 2003). Microbial biomass phosphorus (MBP) plays a key role in maintaining P availability in soil, particularly for highly weathered and P deficient soils. Soil MBP mediates P transformations between organic and inorganic pools, and is a major source of labile P in soil (Tate, 1984; Oberson et al., 2001).

Understandings on mineral phosphorus fractions are necessary to develop sustainable P management practices. The chemistry of P in soils is complex, and several fractionation procedures have been developed to quantify the various inorganic and organic forms of P in soils.

Soil P belongs to two broad groups: organic and inorganic. Organic P is found in plant residues, manures, and microbial tissues. Inorganic forms of soil P, which include iron-P (Fe-P), aluminum-P (Al-P), calcium-P (Ca-P), and occluded-P (Occl-P) (Peterson and Corey, 1963). Kuo (1996) presented a fractionation scheme that incorporated the original Chang and Jackson (1957) fractionation procedure and most of the subsequent modifications. Soils low in organic matter may contain less than 3% of their total P in the organic form, but high organic matter (OM) soils may contain 50% or more of their total P content in the organic form (Griffith, 2011).

Our hypothesis is that combining organic and inorganic nutrient sources may provide an efficient use of resources for maintaining higher activity of microbial biomass P and available pool of P. However, very little information is available at present on soil microbial activity and fractions of P in soils treated with rock phosphate enriched compost (RPEC) and chemical fertilizers and their effect on nutrient availability to crops.

MATERIALS AND METHODS

Site descriptions

The experimental area represents Indo-Gangetic plain and belongs to Mehrauli soil series of order Inceptisols. A field experiment was conducted during winter (*rabi*) season of 2009-2010 on wheat and monsoon (*kharif*) season of 2010 on soybean at the research farm of Indian Agricultural Research Institute (IARI), New Delhi, India. The soil of experimental site is sandy loam in nature and climate is semi-arid subtropical region showing hot summers (May-June) and cold winters (December-January) with an annual average maximum and minimum temperature of 40.5 and 6.5°C, respectively, and the average annual rainfall of 788 mm occurring mostly during the months of July to September. Before start of experiment initial soil samples were collected from surface (0-15 cm) and sub-surface soil (15-30 cm). Physico-chemical and biological properties of soil are presented in Table 1.

Experimental design and treatment details

The field experiment was laid out in a randomized block design with three replications having a plot size of 5.0 m × 4.5 m. The following

Table 1. Initial physico-chemical and biological properties of the experimental soil.

Parameter	Soil depth		Reference
	0-15 cm	15-30 cm	
Mechanical analysis			Bouyoucos (1962)
Sand (%)	56.4	56.3	
Silt (%)	25.0	26.0	
Clay (%)	18.6	17.7	
Texture	Sandy loam	Sandy loam	
CEC [cmol $_{(p+)}$ kg^{-1} soil]	11.68	6.7	Jackson (1973)
pH_w (1:2.5)	8.0	8.0	Jackson (1973)
EC (dS m^{-1})	0.50	0.45	Jackson (1973)
Olson-P (kg ha^{-1})	22.1	18.1	Olsen et al. (1954)
Acid phosphatase (µg PNP g^{-1} h^{-1})	34.2	27.4	Tabatabai and Bremner (1969)
Alkaline phosphatase (µg PNP g^{-1} h^{-1})	120.1	70.8	Tabatabai and Bremner (1969)

Table 2. Characteristics of rock phosphate enriched compost (RPEC) prepared by using rice straw, rock phosphate and *Aspergillus awamori.*

Parameters	Enriched compost
pH_w (1:5)	8.0 ± 0.09
Moisture %	7.92 ± 0.18
CEC [cmol(p+) kg^{-1} compost]	200.0 ± 14.0
Total carbon (%)	25.7 ± 0.29
Total N (%)	1.0 ± 0.07
C/N	25.7 ± 0.02
Total P (%)	3.2 ± 0.01
Water soluble P (WSP %)	0.031 ± 0.03
Citrate soluble P (CSP %)	1.98 ± 0.39
Total K (%)	1.6 ± 0.11
Total S (%)	0.61 ± 0.03
Total Ca (%)	2.83 ± 0.05
Total Mg (%)	1.99 ± 0.08
Total Fe (%)	0.22 ± 0.01
Total Mn (mg kg^{-1})	345.0 ± 15.0
Total Cu (mg kg^{-1})	55.0 ± 0.57
Total Zn (mg kg^{-1})	171.0 ± 14.0
Microbial parameters	
Microbial biomass carbon (g kg^{-1})	4.70 ± 0.24
Dehydrogenase (µg TPF g^{-1} h^{-1})	511.0± 1.00
Acid phosphatase (µg PNP g^{-1} h^{-1})	863.0 ± 37.0
Alkaline phosphatase (µg PNP g^{-1} h^{-1})	2070.0 ± 55.0

treatments were used for conducting the present field experiment. These are: T_1: Control; T_2: Recommended dose of NPK fertilizers (100% RDF); T_3: Rock phosphate enriched compost (RPEC) at the rate of (5 t ha^{-1}; T_4: 50% RDF + Rock phosphate enriched compost (RPEC) at 5 t ha^{-1}. Wheat was grown as the first crop and soybean was grown as the succeeding crop. Recommended dose of NPK fertilizers applied to wheat were: 120:60:60 N, P_2O_5, and K_2O respectively. Urea and diammonium phosphate (DAP) were used as the source of nitrogen, while DAP and muriate of potash (MOP) were used as the source of phosphorus and potassium, respectively. These are collectively called recommended dose of NPK (RDF) fertilizers as per the crop requirement. The whole quantities of compost and fertilizer materials were applied to wheat before last ploughing and soybean was grown in the residual fertility.

Preparation of Rock phosphate enriched compost (RPEC)

Rock phosphate enriched compost (rice straw + Udaipur rock phosphate at 4% P + *Aspergillus Awamori*) was prepared in bulk and used for the present field experiments. Rice straw was mixed with required quantities of rock phosphate and *Aspergillus awamori* at 50 g fresh mycelia per 100 kg of rice straw. A uniform dose of urea solution (0.25 kg N per 100 kg of rice straw), fresh cow dung (5 kg per 100 kg of rice straw), *Trichoderma viride* (50 g fresh mycelia per 100 kg of rice straw) and *Aspergillus awamori* (50 g fresh mycelia per 100 kg of rice straw) was introduced into each composting mass to reduce C/N ratio, as natural inoculums, hasten the composting and P solubilise respectively. The composting was carried out in pots having 100 L capacity. Turning was done by manually at monthly intervals to provide adequate aeration. Moisture content (50 to 60% of field capacity) was maintained throughout the composting period. Composting was continued for 120 days.

Compost analysis

At maturity, fresh compost samples were collected after 120 days of composting and analyzed for total nutrients content. Total nutrient contents were determined as per the standard procedure (Jackson, 1973). Bio-available P consisting of water soluble P (WSP) and neutral *1 N* ammonium citrate soluble P (CSP) was determined as per the procedure outlined by Fertiliser (Control) Order (FCO, 1985). Microbial biomass carbon was determined by chloroform fumigation incubation method as outlined by Jenkinson and Powlson (1976), while dehydrogenase activity was determined by Klein et al. (1971) and acid and alkaline phosphatase by the method as outlined by Tabatabai and Bremner (1969). Chemical and biological characteristics of enriched compost are presented in Table 2.

Post harvest soil analysis

The plot-wise soil samples were collected from surface (0-15 cm) and sub-surface (15 -30 cm) soil after the harvest of wheat and

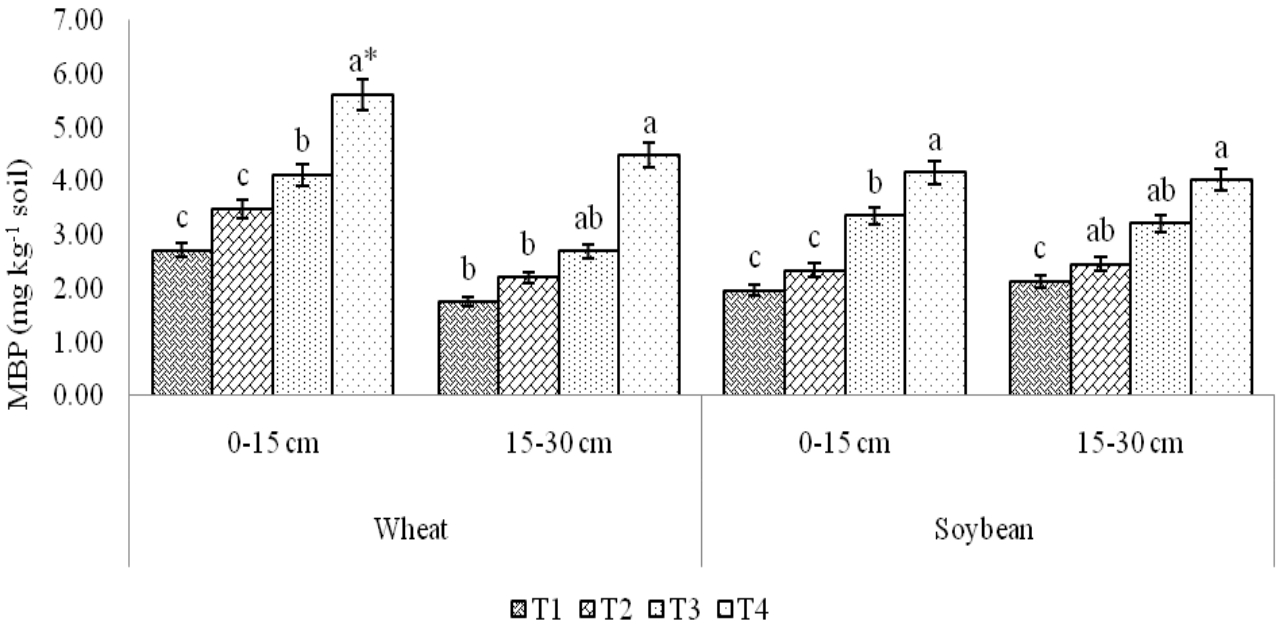

Figure 1. Microbial biomass phosphorus (MBP) under wheat and soybean (mg kg^{-1} soil) as affected by RPEC vis-a-vis and chemical fertilizers .T_1, Control; T_2, recommended dose of NPK fertilizers (100% RDF); T_3, Rock phosphate enriched compost (RPEC) at 5 t ha^{-1}; T_4, 50% RDF + Rock phosphate enriched compost (RPEC) at 5 t ha^{-1}. *For each parameter, different letters within the same column indicate that treatment means are significantly different at P<0.05 according to Duncan's Multiple Range Test for separation of means.

soybean. Immediately after sampling, a portion of the soil samples (~100 g) were kept at 4ºC in a refrigerator in plastic bags for a few days to stabilize the microbiological activity and analyzed for biological properties. Another portion of soil samples were air-dried in shade ground and passed through 2-mm sieve using a wooden pestle and analyzed for Olsen-P (Olsen et al., 1954). Soil phosphorus was fractionated into various inorganic fractions by modified P fractionation scheme of Peterson and Corey (1963). Microbial biomass phosphorus (MBP) was determined by fumigation method as given by Brookes et al. (1982). Acid and alkaline phosphatase activities were determined by the method as outlined by Tabatabai and Bremner (1969).

Statistical analysis

Data generated from the field experiments were subjected to the statistical analyses of appropriate variance to the experimental design. Data were analysed by Duncan's multiple range tests with a probability *P*<0.05 Duncan, 1995). Least significant differences (LSD) between means were calculated using the SPSS program (SPSS version 16.0; SPSS, Inc., Chicago, IL, USA).

RESULTS

Microbial biomass phosphorus (MBP)

Microbial biomass phosphorus (MBP) significantly increased due to integrated use of RPEC and chemical fertilizers (Figure 1) in surface soil (0-15 cm) after wheat harvest. However, no significant differences were found between T_2 and T_3. The values of MBP in surface soil varied from 2.7 mg kg^{-1} in unfertilized control to 5.6 mg kg^{-1} in treatment T_4 after wheat harvest. Unfertilized control plot (T_1) was statistically at par with treatment that received 100% RDF (T_2) in surface soil after soybean harvest. Significant (*P*<0.05) build-up in microbial P due to combined use of inorganic fertilizers and RPEC which clearly indicates the beneficial effect of integrated nutrient management for enhancing microbial P in surface soil after wheat and soybean.

Acid phosphatase

Data in Figure 2 shows that combined use of RPEC along with chemical fertilizers significantly increased the acid phosphatase activity than that of the unfertilized control plot in surface and sub-surface soil after wheat harvest. The lowest value of 29.7 µg PNP g^{-1} soil h^{-1} of acid phosphatase was found in unfertilized control plot which increased to 72.9 µg PNP g^{-1} soil h^{-1} in treatment T_4 in surface soil after wheat.

Data showed no significant differences in acid phosphatase activity under treatments receiving alone chemical fertilizers and RPEC after soybean harvest in both the soil surface (Figure 2). It is evident that acid phosphatase activity ranged from 23.4 to 49.7 and 21.9 to 47.8 µg PNP g^{-1} soil h^{-1} in surface and sub-surface soil respectively, after soybean harvest; the lowest being with unfertilized control plot (T_1).

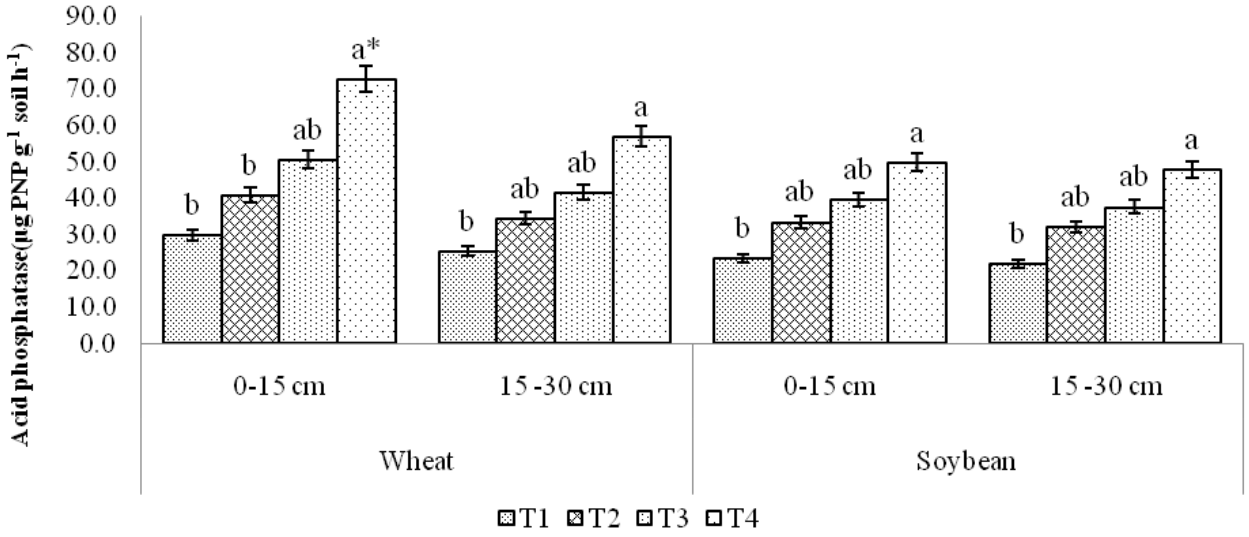

Figure 2. Acid phosphatase activity as affected by RPEC vis-a-vis chemical fertilizers after wheat and soybean harvest .T_1, Control; T_2, recommended dose of NPK fertilizers (100% RDF); T_3, Rock phosphate enriched compost (RPEC) at 5 t ha^{-1}; T_4, 50% RDF + Rock phosphate enriched compost (RPEC) at 5 t ha^{-1}. *For each parameter, different letters within the same column indicate that treatment means are significantly different at P<0.05 according to Duncan's Multiple Range Test for separation of means.

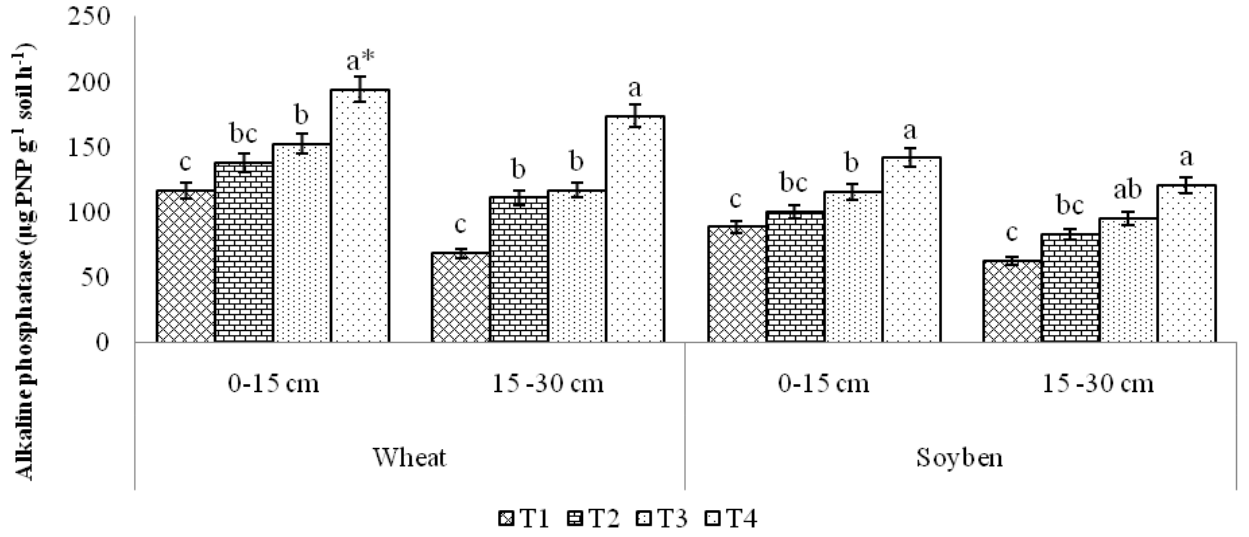

Figure 3. Effect of RPEC and chemical fertilizers on alkaline phosphatase activity after wheat and soybean harvest. T_1: Control; T_2: Recommended dose of NPK fertilizers (100% RDF); T_3: Rock phosphate enriched compost (RPEC) at 5 t ha^{-1}; T_4: 50% RDF + Rock phosphate enriched compost (RPEC) at 5 t ha^{-1}.*For each parameter, different letters within the same column indicate that treatment means are significantly different at P<0.05 according to Duncan's Multiple Range Test for separation of means.

Alkaline phosphatase

There was no significant difference between unfertilized control (T_1) and use of 100% RDF (T_2) in surface soil after wheat harvest (Figure 3). Data pertaining to alkaline phosphatase activity after wheat and soybean clearly illustrated that application of RPEC improved in alkaline phosphatase activity over unfertilized control plot in surface as well as sub surface soil. Significant change in alkaline phosphatase activity was found in treatment receiving conjoint use of RPEC along with chemical fertilizers in surface as well as sub-surface soil after

Table 3. Effects of RPEC and chemical fertilizers on sequential fractionation of P (mg kg^{-1}) after wheat and soybean harvest.

Treatments	Surface soil (0-15 cm)					Sub-surface soil (15-30 cm)				
	After wheat harvest									
	Saloid-P	Fe -P	Al-P	Ca-P	Ocl-P	Saloid-P	Fe -P	Al-P	Ca-P	Ocl-P
T1	9.8^{b*}	13.8^{b}	21.7^{b}	149.5^{c}	14.6^{c}	9.1^{c}	21.2^{a}	15.3^{c}	107.4^{c}	15.7^{c}
T2	20.9^{a}	25.0^{a}	23.6^{b}	186.4^{bc}	15.6^{c}	17.2^{b}	25.0^{a}	$19.4b^{c}$	189.0^{b}	19.7^{bc}
T3	19.6^{a}	25.6^{a}	20.4^{b}	210.1^{ab}	23.9^{b}	17.8^{b}	24.9^{a}	25.3^{ab}	199.6^{b}	21.1^{b}
T4	23.6^{a}	34.1^{a}	34.2^{a}	244.4^{a}	32.5^{a}	20.7^{a}	27.9^{a}	29.6^{a}	230.7^{a}	26.8^{a}
LSD (P=0.05)	3.99	10.3	4.9	36.0	6.8	2.5	8.8	8.4	12.0	3.8
After soybean harvest										
T1	8.7^{b}	15.2^{d}	17.3^{c}	94.0^{c}	13.5^{b}	8.4^{c}	10.0^{d}	16.6^{c}	88.8^{c}	13.0^{c}
T2	16.0^{a}	25.0^{c}	28.8^{b}	132.4^{bc}	17.3^{b}	14.0^{b}	19.0^{c}	29.8^{b}	124.9^{b}	16.3^{b}
T3	16.6^{a}	28.3^{b}	30.4^{ab}	175.9^{ab}	17.3^{b}	12.7^{ab}	27.7^{b}	26.3^{b}	144.3^{b}	18.3^{ab}
T4	19.8^{a}	33.0^{a}	36.0^{a}	222.8^{a}	33.7^{a}	16.5^{a}	31.5^{a}	36.1^{a}	197.7^{a}	20.6^{a}
LSD (P=0.05)	3.9	2.5	5.3	56.1	9.3	2.80	3.48	5.80	21.3	2.9

T_1, Control; T_2, Recommended dose of NPK fertilizers (100% RDF); T_3, Rock phosphate enriched compost (RPEC) at 5 t ha^{-1}; T_4, 50% RDF + Rock phosphate enriched compost (RPEC) at 5 t ha^{-1}. *For each parameter, different letters within the same column indicate that treatment means are significantly different at P<0.05 according to Duncan's Multiple Range Test for separation of means.

soybean harvest than 100% RDF.

Sequential fractionation of P

Data pertaining to the P fractions were affected differentially due to nutrient management practices using RPEC and chemical fertilizers over the unfertilized plot (T_1) after the wheat harvest (Table 3). Application of 100% RDF maintained significantly (*P*<0.05) higher amount of saloid-P, Fe-P, over unfertilized control plot in surface soil after wheat harvest. Combined use of chemical fertilizers and RPEC maintained significantly higher amount of all P fractions namely, saloid–P, Al–P, Ca–P and Occl–P in surface soil as compared to unfertilized control plot after wheat.

The residual effect of RPEC and chemical fertilizers on Saloid-P, Fe-P and Al-P fractions after soybean harvest (Table 3) were higher than the unfertilized plot (T_1) in both surfaces. Treatment that received RPEC + 50% RDF (T_4) maintained significantly highest amount of all fractions such as saloid–P, Fe–P, Al–P, Ca–P and occluded-P than unfertilized plot in surface and sub-surface soil. However, saloid-P was at par with treatment T_2, T_3 and T_4. The relative distribution of P fractions after soybean followed the same trend as in first crop of wheat that is in the order of saloid–P < Fe–P < Al–P < occluded–P < Ca–P in increasing order.

Olsen-P

Application of rock phosphate enriched compost (RPEC) as well as recommended dose of fertilizers applied either alone or in combination maintained significantly (*P*<0.05) higher Olsen-P in soil after wheat and soybean harvest (Table 4) in surface (0-15 cm) and sub-surface (15-30 cm) soil depth as compared to the unfertilized control plot (T_1). Treatment T_4 receiving RPEC at 5 t ha^{-1} along with 50% RDF maintained higher values of Olsen-P by 68.8 and 58.7% over control at 0-15 and 15-30 cm soil depth, respectively after wheat harvest.

Similar trend in build-up of available P was maintained after the soybean crop grown on residual fertility with the application of RPEC and chemical fertilizers (Table 4). In general, the values of Olsen-P in soil after soybean are lower as compared to wheat, irrespective of treatments and soil depths. Treatment T_2 maintained significantly

Table 4. Effect of RPEC and chemical fertilizers on Olson-P after wheat and soybean harvest.

Treatments	Wheat		Soybean	
	0-15 cm	15-30	0-15 cm	15-30 cm
T_1	17.1^{d*}	15.6^{b}	15.0^{c}	14.8^{c}
T_2	25.9^{b}	21.2^{a}	22.3^{b}	20.1^{b}
T_3	23.7^{c}	21.5^{a}	21.8^{b}	19.1^{b}
T_4	28.8^{a}	24.7^{a}	25.8^{a}	22.8^{a}
LSD (P=0.05)	1.1	3.9	2.0	2.1

T_1, Control; T_2, Recommended dose of NPK fertilizers (100% RDF); T_3, Rock phosphate enriched compost (RPEC) at 5 t ha^{-1}; T_4, 50% RDF + Rock phosphate enriched compost (RPEC) at 5 t ha^{-1}. *For each parameter, different letters within the same column indicate that treatment means are significantly different at P<0.05 according to Duncan's Multiple Range Test for separation of means.

higher Olsen-P by 48.7 and 35.9% over unfertilized control in surface and sub-surface soil depth, respectively after soybean harvest.

DISCUSSION

Microbial biomass phosphorus (MBP)

In general, the amount of MBP accounts for 2-10% of total P in soil, which is much larger than the percentage for Olsen-P, and annual P flux through the turnover of the microbial biomass is estimated to be much more than in its standing stock, based on previous measurements made in arable, grassland, and forest soils in both temperate and tropical zones (Brookes et al., 1984; Srivastava and Singh, 1988). Oberson et al. (2001) reported that rapid turnover of P in the microbial pool may contribute a major source to the available P pool, as P released from the microbial biomass is highly available to plant uptake, and also the microbial immobilization of inorganic P protects the P from fixation. In the present study, we found a significant build-up in microbial P due to combined use of inorganic fertilizers and RPEC which clearly indicates the beneficial effect of integrated nutrient management in enhancing microbial P in soils after the first crop of wheat in surface as well as sub-surface soil. It is also evident that MBP was significantly increased in surface soil than sub-surface soil after both wheat and soybean harvest. This may be due to higher microbial activity in the surface soil than the sub-surface soil because of higher amounts of organic matter in case of former.

Acid phosphatase

Application of RPEC significantly increased the phosphatase activity over the application of 100% RDF. This is obvious because organic P present in the compost material is mineralized into inorganic P in presence of phosphatase enzyme which is produced by plants and/or microorganisms and is able to hydrolyze organic P into inorganic P. Acid and alkaline phosphomonoesterases and phosphodiesterases are considered as the predominant phosphatases in most types of soil and litter (Tabatabai, 1994). Wu et al. (2007) studied the effects of sewage or compost on soil P turnover in a short-term incubation experiment and evaluated the relationships that may occur between P availability, bacteria densities and phosphatase activities in a degraded Mediterranean soil characterized by low levels in SOM and N and P nutrients.

Alkaline phosphatise

The present field study clearly indicates that alkaline phosphatase activity in the unfertilized plot declined due to high intensive agriculture as in the present wheat–soybean cropping sequence. Application of 100% RDF increased alkaline phosphatise activity than the unfertilized plot in surface as well as sub-surface soil after wheat and soybean harvest. However, conjoint use of RPEC and chemical fertilizers maintained highest activity of alkaline phosphatase in soil than the sole application of compost or chemical fertilizers, indicating their better enzyme activity which in turn, helps in supplying P to crops.

Sequential fractionation of P

It is clearly seen from the data that P fractions in all the fertilized plots increased than the unfertilized plot. This indicates that there is a build-up of all the P fractions in soil due to application of RPEC and chemical fertilizers. However, treatment receiving combined application of chemical fertilizers and RPEC maintained higher amount of all fractions of P *viz*., saloid–P, Fe–P, Al–P, Ca–P and occluded–P in surface as well as sub-surface soil after wheat harvest. Out of these P fractions, the Ca–P was the dominant fraction found in surface as well as sub-surface soil. Saloid–P, which is considered the most important fraction of P in soil for plant growth, decreased in sub-surface soil. There were no significant treatment effects on Fe-P in sub-surface soil after wheat harvest which may be due to alkaline soil pH of the experimental soil where Fe bound P is very low. The availability of P depends on the soil pH as it governs the occurrence and abundance of those metal cations that are prone to precipitate with P ions in the soil solution, namely Ca, Fe and Al. Hence, in neutral to alkaline soils, P ions rather precipitate as dicalcium or octacalcium phosphates, hydroxyl apatite and eventually least soluble apatites (Hinsinger, 2001).

Olsen-P

Significant build–up in available P due to combined use of chemical fertilizers and RPEC clearly indicates the beneficial effect of integrated nutrient management in enhancing available P in soils after the first crop of wheat. This may be explained as during decomposition of OM lot of organic acids, namely citric, oxalic, tartaric, *etc*. are produced, which in turn, enhanced the dissolution of P from RP, thereby increased P availability (Biswas and Narayanasamy, 2006). It was also evident that higher amounts of available P is present in the surface soil compared to sub-surface soil, indicating that the added P were restricted mainly to surface layer (0-15 cm) and lesser amounts of P have been moved to sub-surface layer (15-30 cm). Availability of Olsen-P was increased in plots receiving organic amendments either alone or in combination with 50% RDF over control unfertilized plot it might be due to the release of organically bound P during decomposition of organic matter, solubilization of soil P by

organic acids. This result corroborates the findings of Moharana et al. (2012).

Conclusions

This study clearly indicated that RPEC along with chemical fertilizers maintained higher amount of MBP, phosphatase enzyme activity and P fractions namely, Saloid-P, Fe-P, Al-P,Ca-P and Ocl-P (occluded) than alone the use of 100% RDF after wheat and soyben harvest. Higher concentration of Olsen-P, was registered under treatments receiving integrated use of RPEC along with 50% RDF than the alone use of chemical fertilizers and RPEC in surface and sub-surface soil depth after wheat and soybean harvest. Thus, RPEC could be an alternative and cost effective option in place of costly chemical P fertilizers.

Conflict of interest

Author did not declare any conflict of interest.

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REFERENCES

Bandick AK, Dick RP (1999). Field management effects on soil enzyme activities. Soil Biol. Biochem. 31:1471-1479.

Biswas DR, Narayanasamy G (2006). Rock phosphate enriched compost: an approach to improve low-grade Indian rock phosphate. Bioresour. Technol. 97:2243-2251.

Bouyoucos GJ (1962). Hydrometer method improved for making particle size analysis of soils. Agron. J. 54:464-465.

Brookes PC, Powlson DS Jenkinson DS (1982). Measurement of microbial biomass phosphorus in soil. Soil Biol. Biochem. 14:319-332.

Brookes PC, Powlson DS, Jenkinson DS (1984). Phosphorus in the soil microbial biomass. Soil Biol. Biochem. 16: 169-175.

Chang SC, Jackson ML (1957). Fractionation of soil phosphorus. Soil Sci. 84:133-144.

Chen X, Tang JJ, Fang ZG, Hu S (2002) Phosphate-solubilizing microbes in rhizosphere soils of 19 weeds in southeastern China. J. Zhejiang Univ. Sci. 3:355-361.

Duncan DM (1995). Multiple range and multiple F-test. Biometric, 11: 1-42.

FAI (2011). Fertiliser Statistics 2010–2011. The Fertiliser Association of India, New Delhi.

FAO (2013). *Available at: http://faostat3.fao.org/faostat-gateway/go/to/home/E* (Verified on 15.10.2013).

FCO (1985). The fertiliser (control) order, 1985 and The Essential Commodities Act, 1955. The Fertiliser Association of India, New Delhi.

Griffith B (2011). Efficient Fertilizer Use Manual, 2011 .http://www.back-to basics.net/efu/pdfs/Phosphorus.pdf.

Hinsinger P (2001). Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. Plant Soil, 237:173-195.

Jackson ML (1973). Methods of chemical analysis. Prentice Hall of India (Pvt.) Ltd, New Delhi.

Jenkinson DS, Powlson DS (1976).The effect of biological treatment on metabolism in soil. Fumigation with chloroform. Soil Biol. Biochem. 8: 167-177.

Klein DA, Loh TC, Goulding RL (1971). A rapid procedure to evaluate dehydrogenase activity in soil of low organic matter. Soil Biol. Biochem. 3:385-387.

Kuo S (1996).Phosphorus. In Methods of Soil Analysis; Sparks, D.L. (ed.); ASA–SSSA: Madison, Wisconsin, Agron. 9.

Meena MD, Biswas DR (2013). Residual effect of rock phosphate and waste mica enriched compost on yield and nutrient uptake by soybean. Legume Research, 36:406-413.

Moharana PC, Sharma BM, Biswas DR, Dwivedi BS, Singh RV (2012). Long-term effect of nutrient management on soil fertility and soil organic carbon pools under a 6-year-old pearl millet–wheat cropping system in an Inceptisol of subtropical India. Field Crops Res. 136:32-41.

Nishanth D, Biswas DR (2008). Kinetics of phosphorus and potassium release from rock phosphate and waste mica enriched compost and their effect on yield and nutrient uptake by wheat *(Triticum aestivum).* Bioresour. Technol. 99:3342-3353.

Oberson A, Friesen DK, Rao IM, Bühler S, Frossard E (2001). Phosphorus transformations in an Oxisol under contrasting land-use systems: the role of the soil microbial biomass. Plant Soil. 237:197-210.

Olsen SR, Cole CW, Watanabe FS, Dean LA (1954). Estimation of available phosphorus in soils by extraction with sodium bicarbonate (U.S. Department of Agriculture Circular 939). Washington, D.C. U.S. Government Printing Office.

Ouedraogo E, Mando A, Zombre NP (2001). Use of compost to improve soil properties and crop productivity under low input agricultural system in West Africa. Agric. Ecosyst. Environ. 84:259-266.

Pan C, Liu C, Zhao H, Wang Y (2013). Changes of soil physico-chemical properties and enzyme activities in relation to grassland salinization. Eur. J. Soil Biol. 55:13-19.

Peterson GW, Corey RB (1963). A modified Chang-Jackson procedure for routine fractionation of inorganic soil phosphates. Soil Sci. Soc. Am. J. 30:563-565.

Srivastava SC, Singh JS (1988). Carbon and phosphorus in the soil biomass of some tropical soils of India. Soil Biol. Biochem. 20:743-747.

Tabatabai MA (1994). Soil enzymes. In: Weaver RM, Angle S, Bottomley P, Bezdicek D, Smith S, Tabatabai A, Wollum, A. (eds) Methods of soil analyses. Part 2. Microbiological and biochemical properties. Soil Sci. Soc. Am. J. Madison, Wis. pp. 775-833.

Tabatabai MA, Bremner JM (1969). Use of P-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol. Biochem. 1:301-307.

Tarafdar JC, Jungk A (1987). A Phosphatase activity in the rhizosphere and its relation to the depletion of soil organic phosphorus. Biol. Fertil. Soil. 3:199-204.

Tate KR (1984). The biological transformation of phosphorus in soil. Plant Soil, 76:245-256.

Van Soest, PJ. (2006). Review - rice straw, the role of silica and treatments to improve quality. Anim. Feed Sci. Technol. 130: 137-171.

Vance CP, Uhde-Stone C, Allan DL (2003). Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. New. Phytol. 157:423-447.

Wu J, Huang M, Xiao HA, Su YR, Tong CL, Huang DY, Syers JK (2007). Dynamics in microbial immobilization and transformations of phosphorus in highly weathered subtropical soil following organic amendments. Plant Soil. 290:333-342.

Yadvinder-Singh, Bijay-Singh, Timsina J (2005). Crop residue management for nutrient cycling and improving soil productivity in rice-based cropping systems in the tropics. Adv. Agron. 85:269-407.

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Full Length Research Paper

Antimicrobial activity of volatile organic compounds and their effect on lipid peroxidation and electrolyte loss in *Colletotrichum gloeosporioides* and *Colletotrichum acutatum* mycelia

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Anthracnose is an important plant disease and is the main disease affecting guava (*Psidium guajava*) post-harvest, leading to economic losses and limiting fruit export. Because of the indiscriminate use of fungicides, along with environmental and food safety concerns, alternative methods of disease control are warranted. The yeast *Saccharomyces cerevisiae* produces a mixture of volatile organic compounds (VOCs) that show *in vitro* antimicrobial activity against various phytopathogens, with the compounds 3-methyl-1-butanol (3M1B) and 2-methyl-1-butanol (2M1B) being primarily responsible for this activity. Considering the potential of using VOCs for controlling post-harvest anthracnose, this study aimed to evaluate the *in vitro* effect of 3M1B and 2M1B on the development of *Colletotrichum gloeosporioides* and *C. acutatum*, the causal agents of the disease in guava, and to elucidate the possible modes of action of these antimicrobials. The mycelial growth of the plant pathogens was inhibited similarly by 3M1B and 2M1B, and complete inhibition occurred at doses ≥1 μL·mL^{-1} of air. Exposure of fungi to the VOCs increased the peroxidation levels of membrane lipids, indicating the occurrence of oxidative stress, in addition to increasing the non-selective permeability of the plasma membrane. Therefore, both 3M1B and 2M1B show potential to control *C. gloeosporioides* and *C. acutatum*.

Key words: anthracnose, inhibition, plasma membrane peroxidation, post-harvest, volatile compounds.

INTRODUCTION

Anthracnose is a disease caused by *Colletotrichum* spp. that impacts the growth of guava (*Psidium guajava* L.) in all guava-producing countries, causing considerable post-harvest losses and limiting fruit export. In the absence of

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control measures, the disease incidence can reach 70 - 100% (Lima Filho et al., 2003).

Although the causal agent of anthracnose in guava is globally accepted to be *C. gloeosporioides* (teleomorph: *Glomerella cingulata*) (Pandey, 1988; Menezes and Hanlin, 1996), there are reports of isolates of *C. acutatum* (teleomorph: *G. acutata*) causing fruit disease in India (Das and Bora, 1998) and Brazil (Peres et al., 2002). Therefore, guava anthracnose can be caused by single or multiple infections of these two fungal pathogens. Among fruits, where the symptoms are more severe, penetration of *Colletotrichum* sp. occurs in unripe fruits in the field. The pathogen can survive in quiescent form, and symptoms can occur at post-harvest. In severe cases, the fruits become mummified or rotten. These symptoms significantly compromise the market for fresh fruits.

The use of only a limited range of fungicidal and fungistatic compounds is allowed at post-harvest because of the risk of a residual effect. To date, no products have been registered for use in guava post-harvest in Brazil. In addition, knowledge of the potential impacts of traditional control practices on the environment and human health has stimulated the search for alternative control methods. In this context, biofumigation of fruits by using microorganisms that produce volatile organic compounds (VOCs) with antimicrobial activity or fumigation by using artificial mixtures of VOCs in closed chambers may be a viable alternative for controlling post-harvest pathogens. In addition to the treatment of fruits and vegetables, VOCs can be used to control pathogens associated with seeds and to fumigate contaminated soils (Strobel, 2011).

VOCs produced by microorganisms are metabolic compounds that are released as gases or exhibit a high vapor pressure and are released from cells under normal conditions (Tarkka and Piechulla, 2007). These compounds generally have a low molecular weight and are active at low concentrations, and can be categorized into different chemical classes (Wheatley, 2002). *Muscodor albus* is a known producer of bioactive VOCs, and the use of the organism itself or an artificial mixture of VOCs it produces has shown potential in controlling a wide range of post-harvest plant pathogens associated to soil, stored seeds, insect pests and plant nematodes (Strobel, 2011). Fialho et al. (2011) reported the potential use of *Saccharomyces cerevisiae* strains for the production of fungistatic VOCs. These compounds inhibited the mycelial growth of *Guignardia citricarpa*, the causal agent of citrus black spot, by up to 87%, in addition to controlling the white mold caused by *Sclerotinia sclerotiorum* in stored bean seeds. The main compounds responsible for the observed inhibition were identified as 3-methyl-1-butanol (3M1B) and 2-methyl-1-butanol (2M1B).

Little is known about the mode of action of antimicrobial VOCs. However, there is evidence that they cause DNA damage in the target organism (Mitchell et al., 2010) and alter the expression of enzymes associated to morphogenesis (Wheatley, 2002), which may affect growth. Moreover, they may alter the properties of cell membranes and trigger oxidative stress. Understanding these mechanisms of action is essential for elucidating the relationship of VOCs with microorganisms and for developing safe fumigants for the control of post-harvest diseases (Fialho et al., 2014). In this context, the objectives of the present study were to evaluate the *in vitro* effect of the VOCs 3M1B and 2M1B, isolated from *S. cerevisiae*, on the development of *C. gloeosporioides* and *C. acutatum* and to elucidate the possible modes of action involved in the observed inhibition, such as changes in membrane permeability and lipid peroxidation resulting from oxidative stress.

MATERIALS AND METHODS

Colletotrichum sp. isolates

C. gloeosporioides and *C. acutatum* were isolated from lesions in symptomatic guava fruits and grown in potato dextrose agar (PDA) at 25°C under a 12-h photoperiod. The cultures were deposited in the Department of Plant Pathology and Nematology at ESALQ-USP, Piracicaba, São Paulo, Brazil.

Antifungal activity

The VOCs 3M1B and 2M1B were purchased from Sigma-Aldrich (USA).

For the antimicrobial activity assays, commercially available two-section divided polystyrene plates (90 mm x 15 mm, 60 mL volume) were used. On one side of the plate containing 10 mL of PDA medium, a 5-mm culture disc containing pathogen mycelia was applied aseptically. On the opposite side of the plate, different volumes (10, 25, 50, and 75 µL) of 3M1B and 2M1B were added to a cotton ball to yield final concentrations of 0.2, 0.5, 1.0, and 1.5 $\mu L \cdot mL^{-1}$ of air, respectively. The compounds 3M1B and 2M1B were evaluated individually or using a mixture ratio of 1:1 (v/v). After the addition of these compounds, the plates were immediately sealed with plastic film and incubated at 25°C under a 12-h photoperiod. The control plates contained the pathogen without the VOCs.

Mycelial growth was assessed when the colonies on the control plates reached the plate edges (72 h), after averaging two diametrically opposed measures of the colony. The data were used to calculate the MIC_{50}, which is the minimum inhibitory concentration capable of inhibiting 50% of pathogen growth. Upon completion of the experiment, the culture discs containing mycelia (initial inoculum) were transferred to plates containing PDA medium without VOCs to assess pathogen viability.

Assessment of electrolyte leakage

Commercially available two-section divided polystyrene plates were used for these assays. On one side of the plate, containing 10 mL of PDA medium, cellophane was aseptically applied to the surface of the medium and a 5-mm culture disc containing pathogen mycelia was transferred on top of the cellophane. The plates were sealed with plastic film and incubated at 25°C under a 12-h photoperiod. After 72 h, 50 µL of 3M1B and 2M1B (1.0 $\mu L \cdot mL^{-1}$ of air), either individually or using a mixture ratio of 1:1 (v/v), was transferred to a cotton ball on the opposite side of the plate. The

plates were sealed with plastic film and incubated for 72 h. The control plates contained the pathogen without the VOCs.

The mycelium was collected, weighed, and transferred to 110-mL plastic cups containing 10 mL of a 0.2 M sucrose solution. For the positive control, 10 µL of the anionic detergent Triton X-100 was added to the sucrose solution. After 60 min, the solutions were transferred to test tubes, and their electrical conductivity was measured with a conductivity meter; the results were expressed as $mS.g^{-1}$ of fresh mycelium.

Assessment of lipid peroxidation

Lipid peroxidation was assessed based on the production of metabolites (primarily malondialdehyde - MDA) that were reactive to 2-thiobarbituric acid (TBA), as described by Heath and Packer (1968) and Cakmak and Host (1991). Fungi were grown as described above by using a dose of 1.0 $\mu L \cdot mL^{-1}$ of air of 3M1B and 2M1B, either individually or using a mixture ratio of 1:1 (v/v). The cellophane containing the mycelium was collected and weighed, and 300 mg of this material was homogenized in 1.3 mL of 0.1% (w/v) trichloroacetic acid (TCA) by using a mortar and pestle in the presence of liquid nitrogen. After centrifugation at 10,000 *g* for 15 min at 4°C, an aliquot containing 500 µL of the supernatant was added to test tubes containing 1.5 mL of 0.5% TBA (w/v) in 20% TCA (w/v). The test tubes were subsequently incubated in a water bath at 90°C for 20 min and cooled in an ice bath. The samples were then centrifuged at 16,000 *g* for 4 min, and the absorbance was measured at 535 nm. The concentration of the MDA-TBA mixture was measured using the molar extinction coefficient of MDA, which corresponds to 155 $mM^{-1} \cdot cm^{-1}$, and data were expressed as $nmol.g^{-1}$ of fresh mycelium.

All experiments were carried out under a completely randomized design, with four replicates per treatment, and each replicate consisted of a single plate.

RESULTS

Mycelial growth

The exposure of *C. gloeosporioides* and *C. acutatum* to different concentrations of 3M1B and 2M1B significantly inhibited mycelial growth (Figure 1). The degree of inhibition caused by these two compounds was similar for the two fungal species and was proportional to the increase in the concentration. Growth inhibition occurred at concentrations ≥0.2 $\mu L \cdot mL^{-1}$ of air and varied between 15 and 20%. In addition, 100% inhibition was observed at concentrations ≥1.0 $\mu L \cdot mL^{-1}$ of air.

No synergistic effect on fungal inhibition was observed between the two compounds compared to the inhibition exhibited by the isolated compounds (Figure 2). The 3M1B/2M1B mixture also promoted fungal inhibition starting at the lowest concentration tested (0.2 $\mu L \cdot mL^{-1}$ of air), and maximal inhibition was achieved at ≥1.0 $\mu L \cdot mL^{-1}$ of air. The MIC_{50} values were also measured and differed significantly only for the comparisons of *C. gloeosporioides* grown in the presence of the 3M1B/2M1B mixture with the other treatments (Table 1).

Based on the results shown in Figures 1 and 2 and in Table 1, no significant difference in pathogen inhibition was observed between the treatments in which 3M1B and 2M1B were applied individually. However, when considering the MIC_{50} values, *C. acutatum* was more sensitive than *C. gloeosporioides* when exposed to the VOCs mixture at the 1:1 ratio (v/v), indicating that the compounds at this ratio exert a synergistic effect. Both pathogens resumed growth when transferred to PDA medium in the absence of the tested VOCs, indicating the fungistatic activity of the VOCs.

It was observed that *C. gloeosporioides* produced a higher biomass compared to *C. acutatum*. However, the two fungi were inhibited similarly when exposed to the VOCs (Figure 3), and no significant difference in their responses was observed when the compounds were applied either individually or as a mixture. The mean percentages of *C. gloeosporioides* and *C. acutatum* inhibition after treatment were 64.9 and 55.4%, respectively.

Electrolyte leakage

The mycelium of *C. gloeosporioides* exposed to the VOCs showed electrolyte losses, based on the observed increase in electrical conductivity, by an average of 66% compared to control fungi (Figure 4). Similarly, exposure to the VOCs caused electrolyte losses in *C. acutatum*, as the electrical conductivity of the exposed fungi was three times higher than that of the control. No significant differences in electrolyte losses were observed when the two compounds were tested individually or as mixture.

Lipid peroxidation

Regarding lipid peroxidation, Figure 5 shows that the levels of MDA in *C. gloeosporioides* mycelium increased approximately 260% when the fungus was exposed to the VOCs. Similarly, lipid peroxidation in *C. acutatum* increased upon exposure to the VOCs, though to a lesser extent; 233% on average compared to the control.

DISCUSSION

There is a high potential in the use of volatile metabolites produced by microbes to control plant pathogens in fruits, grains, and stored seeds (Strobel, 2011). Preliminary studies have shown the inhibition potential of increasing doses of an artificial mixture of VOCs isolated from *S. cerevisiae* against the mycelial growth of *G. citricarpa*, with this inhibition reaching up to 88% (Fialho et al., 2010).

According to the authors, this artificial mixture comprised six compounds and exhibited an MIC_{50} of 0.48 $\mu L \cdot mL^{-1}$; the same MIC_{50} was observed herein for *C. gloeosporioides* exposed to the 3M1B/2M1B mixture.

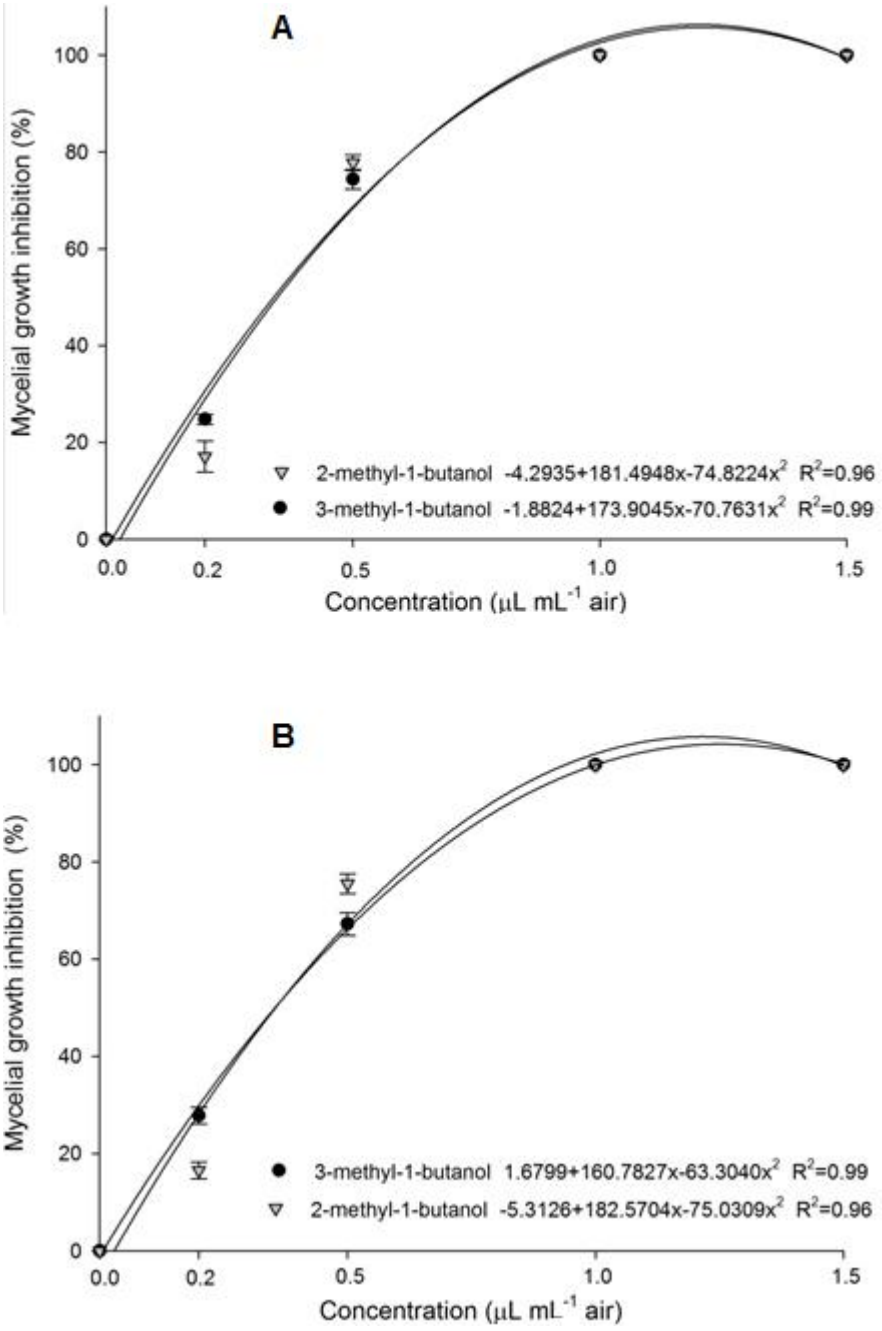

Figure 1. Inhibition of the mycelial growth of *C. gloeosporioides* (A) and *C. acutatum* (B) exposed to the volatile organic compounds 3-methyl-1-butanol (●) and 2-methyl-1-butanol (▽). The mean values of four replicates were calculated as the percentage of inhibition compared to the control without the addition of volatile compounds.

Similar to the results of the present study, the plant pathogens *Botrytis cinerea* and *Penicillium expansum* affecting post-harvest processes were shown to be inhibited by the fungus *Nodulisporium* sp. CF016, when exposed to VOCs for three days (Park et al., 2010). The volatile compounds dimethyl disulfide, dimethyl trisulfide, and acetophenone, isolated from *Streptomyces globisporus,* completely inhibited the growth of the plant pathogen *Penicillium italicum* after five days of exposure to a concentration of 0.1 µL·mL^{-1} of air (Li et al., 2010). In the same way, an artificial mixture of VOCs, consisting of 20 compounds isolated from the fungus *M. albus*, was demonstrated to impair the development of various microorganisms, including *C. gloeosporioides*. The

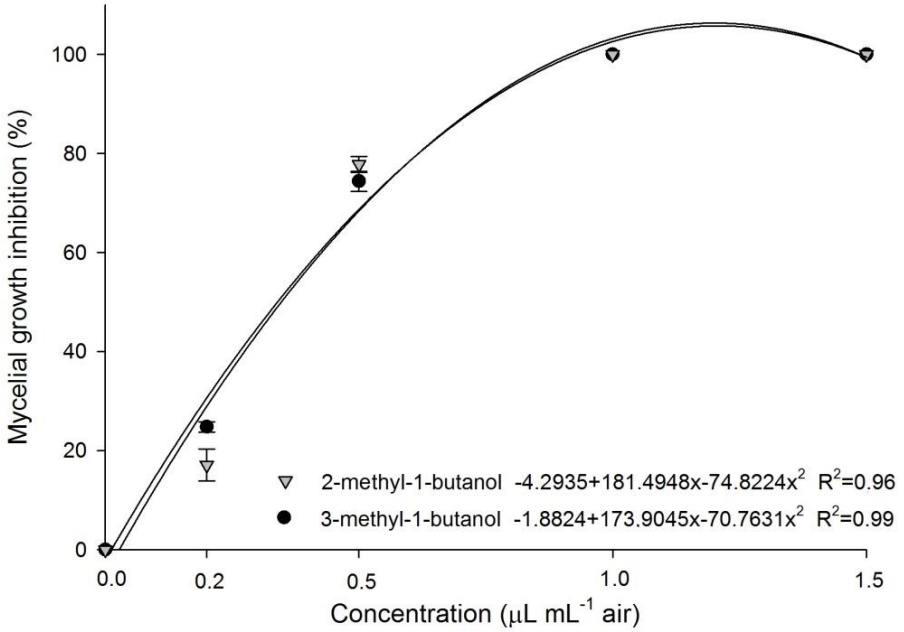

Figure 2. Inhibition of the mycelial growth of *C. gloeosporioides* (●) and *C. acutatum* (▽) grown on PDA medium when exposed to a mixture of the volatile organic compounds 3-methyl-1-butanol and 2-methyl-1-butanol [1:1 ratio (v/v)]. The mean values of four replicates were calculated as the percentage of inhibition compared to the control without the addition of volatile compounds.

Table 1. MIC_{50} for the mycelial growth of *C. gloeosporioides* and *C. acutatum* grown in the presence of the volatile organic compounds 3-methyl-1-butanol (3M1B) and 2-methyl-1-butanol (2M1B).

Pathogen	Treatment	MIC_{50}*	SD
C. gloeosporioides	3M1B	0.33a	0.039
	2M1B	0.34a	0.052
	3M1B:2M1B (1:1, v/v)	0.48b	0.076
C. acutatum	3M1B	0.34a	0.052
	2M1B	0.35a	0.034
	3M1B:2M1B (1:1, v/v)	0.27a	0.057

*$\mu L.mL^{-1}$ of air. The values represent the means of four replicates (mean ± SE). Means followed by the same letter did not differ by the Tukey test ($p \leq 0.05$).

mycelial growth of the pathogen was totally inhibited, although the effect was not lethal at a concentration of 1.2 $\mu L \cdot mL^{-1}$, with a MIC_{50} of 0.3 $\mu L \cdot mL^{-1}$ (Atmosukarto et al., 2005), and similar results were obtained in the present study. Previous studies demonstrating the antimicrobial effect of VOCs produced by bacteria against important pathogens in the post-harvest period also appear promising. The effect of VOCs and secondary metabolites produced by strains of *Bacillus subtilis* on the mycelial growth of *P. digitatum* revealed that exposure of the fungus to VOCs caused inhibition of 30 -70%. However, as observed in the present study, pathogen growth was recovered when the mycelium was cultured in plates containing only PDA medium, demonstrating the fungistatic effect of the compounds (Leelasuphakul et al., 2008).

The mean inhibition of mycelial biomass production observed by using the evaluated VOCs was 64.9% for *C. gloeosporioides* and 55.4% for *C. acutatum*. Similar results were obtained by Humphris et al. (2001). These authors reported that *Trichoderma* species, which produce several VOCs showing inhibitory activity against plant pathogens, also produce 2M1B, and they found that biomass production was decreased by up to 80% in wood-decay fungi when exposed to 2M1B at 2,500 $\mu g \cdot mL^{-1}$ of air. However, in the present study, fungal inhibition with 2M1B was achieved at a concentration of just 1 $\mu g \cdot mL^{-1}$ air.

In contrast to the findings of the present study, when Tunc et al. (2007) investigated the VOCs ethanol and carvacrol, they observed a synergistic effect between these compounds in the inhibition of *P. notatum*. In *M. albus*, the VOCs naphthalene, propanoic acid, and 3M1B were shown to display biological activity against the plant pathogens *Pythium ultimum*, *R. solani*, and *S. sclerotiorum*. The use of artificial mixtures demonstrated that all three compounds had to be present to produce this inhibitory activity, indicating the synergistic effects of

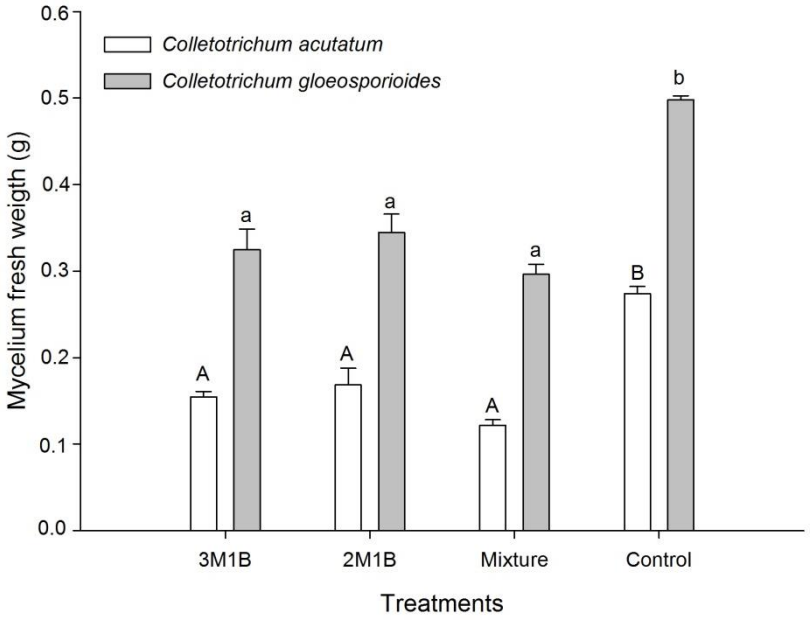

Figure 3. Effect of the volatile organic compounds 3-methyl-1-butanol (3M1B) and 2-methyl-1-butanol (2M1B) and their mixture (1:1 ratio; v/v) at a dose of 1.0 $\mu L \cdot mL^{-1}$ of air on mycelium production by *C. gloeosporioides* and *C. acutatum* after 72 h of exposure. The control plates contained the pathogen without the addition of VOCs. The values represent the means of four replicates (mean ± SE). Means followed by the same letter did not differ by the Tukey test ($p \leq 0.05$).

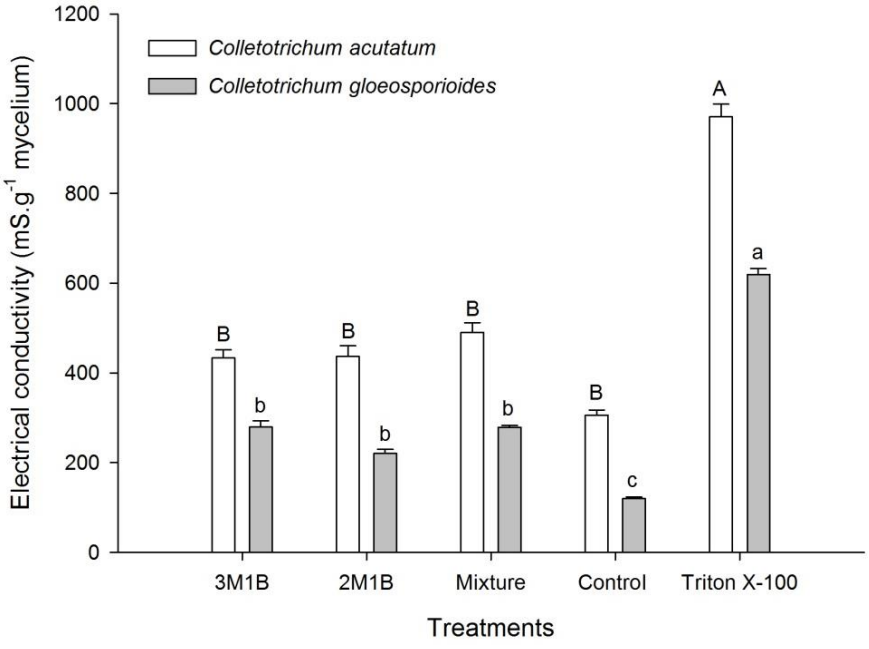

Figure 4. Effect of the volatile organic compounds 3-methyl-1-butanol (3M1B) and 2-methyl-1-butanol (2M1B) and their mixture (1:1 ratio; v/v) at a dose of 1.0 $\mu L \cdot mL^{-1}$ of air on electrolyte loss in *C. gloeosporioides* and *C. acutatum* mycelia after 72 h of exposure. The control and Triton X-100 treatments consisted of the pathogen without the addition of VOCs, and Triton X-100 was added to the sucrose solution at the time of evaluation. The values are the means of four replicates (mean ± SE). Means followed by the same letter did not differ by the Tukey test ($p \leq 0.05$).

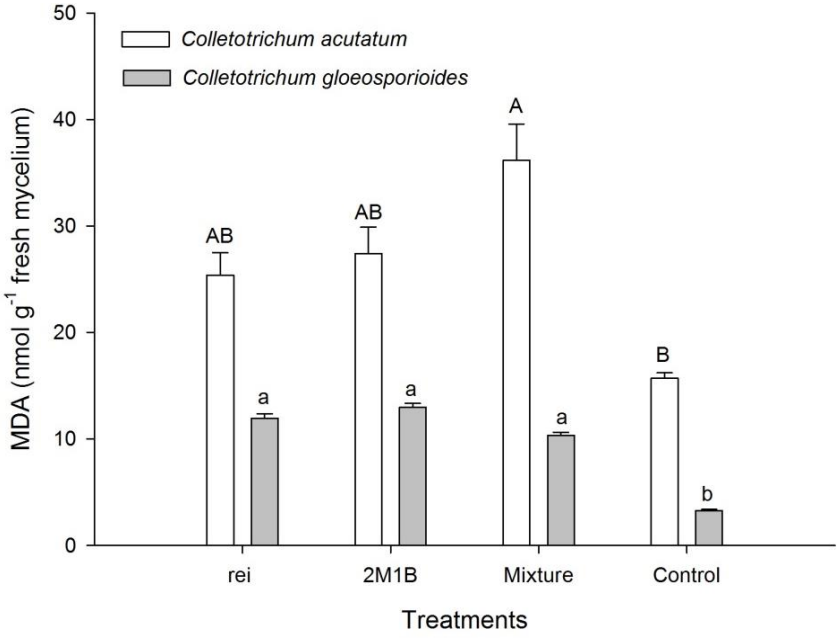

Figure 5. Effect of the volatile organic compounds 3-methyl-1-butanol (3M1B) and 2-methyl-1-butanol (2M1B) and their mixture (1:1 ratio; v/v) at a dose of 1.0 µL·mL^{-1} of air on lipid peroxidation (MDA content) in *C. gloeosporioides* and *C. acutatum* mycelia after 72 h of exposure. The control plates contained the pathogen without the addition of VOCs. The presented values are the means of four replicates (mean ± SE). Means followed by the same letter did not differ by the Tukey test ($p \leq 0.05$).

this mixture (Ezra et al., 2004). In the present study, no synergistic effect of the 3M1B/2M1B mixture was observed, most likely because these compounds are isomers and therefore exert similar effects on plant pathogens.

The mechanisms involved in the antimicrobial activity of 3M1B and 2M1B remain unknown. In this study, exposure of *C. gloeosporioides* and *C. acutatum* to both alcohols resulted in increased electrolyte loss, indicating damage to the plasma membrane, which may have contributed to the impaired fungal development. To the best of our knowledge, this is the first study to evaluate the effect of alcohols on the plasma membrane of *C. gloeosporioides* and *C. acutatum* when in contact with these compounds in the gas phase.

In the case of alcohols, such as ethanol, the mechanisms involved in the antimicrobial activity have been elucidated and appear to be more strongly correlated with physicochemical characteristics than with the interaction with specific receptors on the target cells. Alcohols cause protein denaturation and affect the organization and stability of the lipid bilayer of the plasma membrane. Damage to the plasma membrane results in increased non-selective permeability, which leads to loss of ions and essential metabolites (Ingram and Buttke, 1984; Seward et al., 1996). Both 3M1B and 2M1B are higher aliphatic alcohols and consequently show an increased potential to damage cell membranes compared to less lipophilic substances, such as ethanol, for which high concentrations are needed to exert antimicrobial activity (Heipieper et al., 2000).

Alcohols can interfere with cellular growth, morphogenesis, transport systems, the loss of metabolites and the composition and biosynthesis of lipids. Alcohols cause deleterious effects on the arrangement of lipid components in the plasma membrane of *Escherichia coli* K-12 and inhibit its growth by 30-40%. However, bacterial cultures exposed to ethanol were observed to resume growth after recovery of the composition of the membrane fatty acids (Ingram, 1976). Heipieper et al. (2000) investigated the tolerance and adaptation of membrane fatty acids to ethanol in mutants of *Kluyveromyces lactis* and found that the MIC_{100} for the tested isolates was 8-10% ethanol (w/v).

Reactive oxygen species (ROS), such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), are potent oxidizing agents that can accumulate intracellularly at dangerous levels after exposure to physical and chemical factors such as UV radiation and heavy metals. When antioxidant mechanisms of cellular defense, such as catalase (CAT) and superoxide dismutase (SOD) activities, are not sufficient to detoxify ROS, oxidative stress can occur because ROS can react rapidly and non-specifically with macromolecules, resulting

in DNA mutation, protein oxidation, and lipid peroxidation of cell membranes (Angelova et al., 2005; Heller and Tudzynski, 2011). However, the available data on oxidative stress in organisms exposed to VOCs are limited. In the present study, the levels of MDA, a product of lipid peroxidation, increased in *C. gloeosporioides* and *C. acutatum* exposed to 3M1B and 2M1B, indicating that these VOCs caused an imbalance in the redox state of the fungi, given that lipid peroxidation is a marker of oxidative stress. Hence, lipid peroxidation may have caused the increased cell permeability observed herein because the effects of lipid peroxidation include decreased membrane fluidity and increased permeability to protons, ultimately leading to cell rupture. Furthermore, cytotoxic compounds such as hydroperoxides are formed during this process (Li et al., 2009). Peroxidation may affect the functionality of the cell membrane, resulting in irreversible damage to cellular functions. Therefore, lipid peroxidation may be indicative of plasma membrane damage. Although the growth of *C. gloeosporioides* and *C. acutatum* was shown to be inhibited by alcohols; this effect was not fungicidal. It is possible that these two fungal species employ antioxidant defense mechanisms, such as increased activity of the enzymes CAT and SOD, which can minimize the generation of ROS to some extent. For example, Fialho et al. (2014) observed increased activity of CAT and SOD in the mycelia of *G. citricarpa* exposed to an artificial mixture of VOCs isolated from *S. cerevisiae*. On the other hand, Splivallo et al. (2007) investigated the possible mode of action of VOCs produced by truffles (*Tuber* spp.), including 3M1B, on the growth of the plant *Arabidopsis thaliana*. Although plants and microorganisms are phylogenetically distinct organisms, some of their characteristics are similar, and they may therefore share the same targets of VOCs. Some 8-carbon compounds, such as 1-octen-3-ol and trans-2-octenal, were shown to be the most effective in inhibiting plant growth, inducing the production of ROS (H_2O_2), and increasing the activity of antioxidant enzymes.

Conclusion

Based upon the results of this study, the compounds 3-methyl-1-butanol and 2-metil-1-butanol show potential for controlling *C. gloeosporioides* and *C. acutatum* growth. Changes in pathogen plasma membrane permeability is one of the modes of action involved in the inhibition process. Therefore, the use of these volatiles as fumigants in the post-harvest treatment of guava fruits against anthracnose appears to be possible.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

Angelova MB, Pashova SB, Spasova BK, Vassilev SV, Slokoska LS (2005). Oxidative stress response of filamentous fungi induced by hydrogen peroxide and paraquat. Mycol. Res. 109: 150-158.

Atmosukarto I, Castillo U, Hess WM, Sears J, Strobel G (2005). Isolation and characterization of *Muscodor albus* I-41.3s, a volatile antibiotic producing fungus. Plant Sci. 169: 854-861.

Cakmak I, Horst WJ (1991). Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase and peroxidase activities in root tips of soybean. Physiol. Plant. 83:463-468.

Das M, Bora KN (1988). Ultrastrutuctural studies on infection processes by *Colletotrichum acutatum* on guava fruit. Indian Phytopathol. 51:353-356.

Ezra D, Hess WM, Strobel GA (2004). New endophytic isolates of *Muscodor albus*, a volatile-antibiotic-producing fungus. Microbiology 150:4023-4031.

Fialho MB, Carvalho G, Martins PF, Azevedo RA, Pascholati SF (2014). Antioxidative response of the fungal plant pathogen *Guignardai citricarpa* to antimicrobial volatile organic compounds. Afr. J. Microbiol. Res. 8:2077-2084.

Fialho MB, Moraes MHD, Tremocoldi AR, Pascholati SF (2011). Potential of antimicrobial volatile organic compounds to control *Sclerotinia sclerotiorum* in bean seeds. Pesq. Agropec. Bras. 46:137-142.

Fialho MB, Toffano L, Pedroso MP, Augusto F, Pascholati SF (2010). Volatile organic compounds produced by *Saccharomyces cerevisiae* inhibit the *in vitro* development of *Guignardia citricarpa*, the causal agent of citrus black spot. World J. Microbiol. Biotechnol. 26:925-32.

Heath RL, Packer L (1968). Photoperoxidation in isolated chloplasts. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125:189-1988.

Heipieper HJ, Isken S, Saliola M (2000). Ethanol tolerance and membrane fatty acid adaptation in *adh* multiple and null mutants of *Kluyveromyces lactis*. Res. Microbiol. 151:777-784.

Heller J, Tudzynski P (2011). Reactive oxygen species in phytopathogenic fungi: signaling, development and disease. Annu. Rev. Phytopathol. 49:369-390.

Humphris SN, Wheatley RE, Bruce A (2001). The effects of specific volatile organic compounds produced by *Trichoderma* spp. on the growth of wood decay basidiomycetes. Holzforschung 55:233-237.

Ingram LO (1976). Adaptation of membrane lipids to alcohols. J. Bacteriol. 125:670-678.

Ingram LO, Buttke TM (1984). Effects of alcohols on microorganisms. Adv. Microb. Phys. 25:253-300.

Leelasuphakul W, Hemmaneea P, Chuenchitt S (2008). Growth inhibitory properties of *Bacillus subtilis* strains and their metabolites against the green mold pathogen (*Penicillium digitatum* Sacc.) of citrus fruit. Postharvest Biol. Technol. 48:113-121.

Li Q, Harvey LM, McNeil B (2009). Oxidative stress in industrial fungi. Crit. Rev. Biotechnol. 29: 199-213.

Li Q, Ning GP, Zheng L, Huang J, Li G, Hsiang T (2010). Fumigant activity of volatiles of *Streptomyces globisporus* JK-1 against *Penicillium italicum* on *Citrus microcarpa*. Postharvest Biol. Technol. 58:157-165.

Lima Filho RM, Oliveira SMA, Menezes M (2003). Enzymatic characterization and crossed pathogenicity of *Colletotrichum* spp. associated with postharvest diseases. Trop. Plant Pathol. 28:620-625.

Menezes M, Hanlin RT (1996). Apressoria of Brazilian isolates of *Colletotrichum gloeosporioides* (Penz.) Sacc. causal agent of anthracnose diseases. Braz. J. Microbiol. 27:247-251.

Mitchell AM, Strobel GA, Moore E, Robison R, Sears J (2010). Volatile antimicrobials from *Muscodor crispans*, a novel endophytic fungus. Microbiology 156:270-277.

Pandey RR (1988). Effect of foliar aplications of fungicides on the phylloplane mycoflora and fungal pathogens of guava. J. Phytopathol. 123:52-62.

Park MS, Ahn J, Choi JG, Choi YH, Jang KS, Kim JC (2010). Potential of the volatile-producing fungus *Nodulisporium* sp. CF016 for the control of postharvest disease of apple. Plant Pathol. J. 26:253-259.

Peres NAR, Kuramae EE, Dias MSC, Souza NL (2002). Identification and characterization of *Colletotrichum* spp. affecting fruit after harvest in Brazil. J. Phytopathol. 150:128-134.
Seward R, Willetts JM, Dinsdale MG, Lloyd D (1996). The effects of ethanol, hexan-1-ol, and 2-phenylethanol on cider yeast growth, viability, and energy status; synergistic inhibition. J. Inst. Brew. 102:439-443.
Splivallo R, Novero M, Bertea CM, Bossi S, Bonfante P (2007). Truffle volatiles inhibit growth and induce an oxidative burst in *Arabidopsis thaliana*. New Phytol. 175:417-424.
Strobel G (2011). Muscodor species-endophytes with biological promise. Phytochem. Rev. 10: 165-172.
Tarkka MT, Piechulla B (2007). Aromatic weapons: truffles attack plants by the production of volatiles. New Phytol. 175:381-383.
Tunc S, Chollet E, Chalier P, Preziosi-Belloy L, Gontard N (2007). Combined effect of volatile antimicrobial agents on the growth of *Penicillium notatum*. Int. J. Food Microbiol. 113:263-270.
Wheatley RE (2002). The consequences of volatile organic compound mediated bacterial and fungal interactions. Antonie van Leeuwenhoek 81:357-364.

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Full Length Research Paper

Identification of a chitinase from *Apocheima cinerarius* nucleopolyhedrovirus

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Baculoviruses are important biological control agents against insect pests. In this work, a chitinase from *Apocheima cinerarius* nucleopolyhedrovirus (ApciNPV) as a fusion protein was highly over-expressed in *Escherichia coli* M 15 strain and affinity purified on Ni Sepharose 6 Fast Flow column. The modeling structure of ApciNPV chitinase indicated the protein contains a polycystic kidney 1(PKD1) domain on N-terminal and a characteristic catalytic domain formed α/β triosephosphate isomerase (TIM) barrel fold like other family 18 glycohydrolases. The ApciNPV chitinase displayed both endo- and exo-chitinase activities using fluorescent oligosaccharides. Moreover, assessed by LC_{50} (50% lethal concentration) values, the ApciNPV chitinase showed insecticidal activity against *Apocheima cinerarius*, *Spodoptera exigua*, *Hyphantria cunea*, *Helicoverpa armigera* and *Lymantria dispar*. The results suggest that ApciNPV chitinase may offer a potential application as a new tool to control pest.

Key words: *Apocheima cinerarius* nucleopolyhedrovirus, chitinase, recombinant protein, insecticidal activity.

INTRODUCTION

Chitinases are enzymes that degrade chitin, which have wide-range roles in biocontrol of pathogens and harmful insects (Cohen-Kupiec and Chet, 1998). Baculovirus chitinases are considered responsible for the liquefaction of insect host (Hawtin et al., 1995; Hawtin et al., 1997) and are used as bioinsecticides for insect control. As reported by Rao et al. 2004 (Rao et al., 2004) in *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), the chitinase induced 100% mortality for *Bombyx mori* larvae and a significant increase in the number and size of perforations of the peritrophic membrane (PM). The chitinase from *B. mori* nucleopolyhedrovirus (BmNPV) plays an important role in delaying the cell lysis and decreasing the haemolymph turbidity and the degradation of the body in silkworm larvae (Wang et al., 2005). Furthermore, chitinase and V-cathepsin could together advance the liquefaction of the host after death (Hawtin et al., 1997; Ohkawa et al., 1994; Hom et al., 2000; Slack et al., 1995).

Apocheima cinerarius nucleopolyhedrovirus (ApciNPV) was first isolated and identified by the Institute of Forest Ecology and Environment Conservation, Chinese Academy of Forestry in 1979 (Yu and Wang; 1987; Yu and Wang, 1986). It is a member of the Baculoviridae family, Genus

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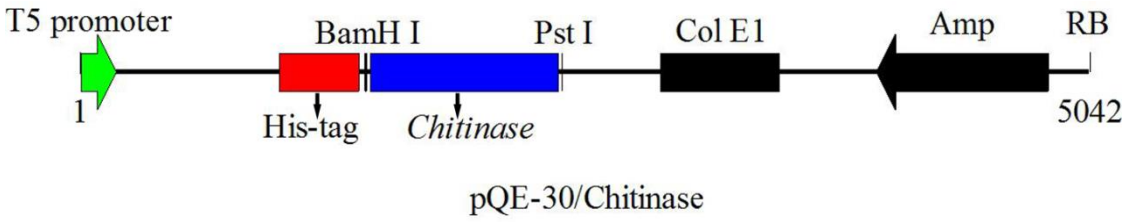

Figure 1. Outline of the pQE 30 expression vector with chitinase from ApciNPV under control of T5 promoter.

of alphabaculovirus. The virus exhibited high virulence against the larvae of *A. cinerarius* (Qi, 2006). In this paper, a gene which encodes a chitinase of ApciNPV is expressed in *Escherichia coli* and its potential use as a biological insecticide is proposed.

MATERIALS AND METHODS

Propagation of viruses

A. cinerarius larvae, ApciNPV strain and artificial diet were provided by the Research Centre of Forest Insect Virus, Chinese Academy of Forestry. ApciNPV was replicated and purified according to the method described by Qu et al. (2011). Briefly, ApciNPV in the concentrations of 3.0×10^7 was used to infect fourth-instar *A. cinerarius* larvae. After 5 days, 30 dead insects were milled and fully homogenized with two volumes phosphate buffer solution (PBS, pH 7.2). To remove insect tissue, the extract was filtered through four layers of gauze and then the filtrate was stirred at 800 rpm for 5 min at 25°C. The crude virus was obtained by centrifugation (8000 rpm, 30 min), and purified in a sucrose gradient of 40-60% (w/w) at 4000 rpm for 30 min at 25°C. The bands containing the virus were collected and washed with sterile water three times by centrifugation at 10000 rpm for 30 min at 4°C.

Purification of viral DNA

The method of isolation of ApciNPV DNA was performed basically according to Wang et al. (Wang et al., 2013). Purified ApciNPV was suspended in 2-3 volumes extraction buffer (0.1 M Na_2CO_3, 0.15 M NaCl and 0.05 M EDTA, pH 10.8) to dissolve the polyhedron matrix. After incubating at 3°C for 1 h, the suspension was adjusted to pH 7.0 with 0.1 M HCl, and SDS (0.5%) and proteinase K (50 µg/mL) were then added successively, and digestion was performed at a temperature of 55°C for 3 h. The solution was extracted with the same volume phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform, respectively. The ApciNPV DNA was precipitated with two volumes of ethanol at -20°C for 2 h, and obtained by centrifugation at 12000 rpm for 10 min at 4°C. The precipitate was dissolved in TE buffer (pH 8.0) and stored at -20°C.

Construction of bacterial expression plasmids

A truncated sequence of *orf38* chitinase gene lacking its C-terminal ER retention motif (HTEL) was amplified from ApciNPV genome (GenBank accession number: FJ914221) using primers P1 (5' -AAAAGGATCCATGCATTGGTGCGTCAACCGCGA-3' and P2 5'-CCCCCTGCAGTTTTATATTAGATTTATAATGT -3') to generate *Bam*H I and *Pst* I restriction sites (underlined) at the 5'end and 3'end, respectively. The 50 µL PCR solution including 40 ng DNA, 0.1 µmol forward and reverse primers and 1× polymerase buffer (containing 15 mM $MgCl_2$, 0.4 mM dNTP, 5U of Expand High Fidelity Taq polymerase). The reaction of PCR operated at 94°C for 5 min and repeated 30 times following 94°C for 30 s, 60°C for 45 s, 72°C for 1 min, and final extension went at 72°C for 10 min. The amplified PCR product was digested using the enzymes of *Bam*H I and *Pst* I, and constructed into the pQE30 vector (Novagen) with 6×his-tag gene and T5 promoter (Figure 1).

Protein production and purification

The ApciNPV chitinase as a recombinant fusion protein with a 6×his-tag was over-expressed in *E. coli* strain M15 (Novagen). Small-scale (3 mL) cells were cultured with a rotary shaker at 37°C until the optical density at 600 nm (OD 600) of 0.6 and the recombinant protein was induced by 0.2 mM IPTG treatment at 16°C for 15 h. The cells were obtained by centrifugation, kept at -20°C for 30 min, and then suspended in FastBreak™ Cell lysis Reagent (Promega). The amount of soluble and insoluble recombinant protein was determined by MagneHis™ Protein Purification System according to the manufacturer's instructions (Promega). Large-scale (1 L) recombinant protein was obtained by adding 0.2 mM IPTG to *E. coli* culture (OD 600=0.6). After induction process for 15 h at 16°C, the cells were harvested and lysed in 80 mL buffer A (20 mM Tris-HCl, 150 mM NaCl, 10 mM Imidazole, pH 7.5), and then sonicated on ice with Sonifier (300W, 3s/2s). After centrifugation, the soluble fraction was adsorbed on Ni Sepharose 6 Fast Flow (GE healthcare) column. The column was equilibrated with buffer A and initially eluted with buffer B (20 mM Tris-HCl, 150 mM NaCl, 20 mM Imidazole, pH 7.5). Adsorbed protein was eluted with buffer C (20 mM Tris-HCl, 150 mM NaCl, 200 mM Imidazole, pH 7.5) and buffer D (20 mM Tris-HCl, 150 mM NaCl, 500 mM Imidazole, pH 7.5), sequentially. The separation and purification of the samples were confirmed by SDS-PAGE (12% gel) and the expected band was visualized staining the gel with Coomassie brilliant blue.

Enzyme activity analysis

The enzyme activity of the recombinant ApciNPV chitinase was analyzed as previously described (Rao et al., 2004; McCreath et al., 1992) with 4-methylumbelliferyl β-D-N,N′ diacetilchitobioside (4MU-$(GluNAC)_2$) and 4- methylumbelliferyl β-D-N, N′,N″ triacetilchito-trioside (4MU-$(GluNAC)_3$) as substrates for the quantified of exo-chitinase and endo-chitinase activities, respectively. For each standard sample, 20 µL McIlvaine's buffer (0.1 M citric acid, 0.2 M dibasic sodium phosphate, pH 5.0) and 5 µL appropriate substrate was mixed and then seven different concentrations (0, 5, 10, 15, 20, 25, 30 µg/µL) of protein were added into each tube. After incubating

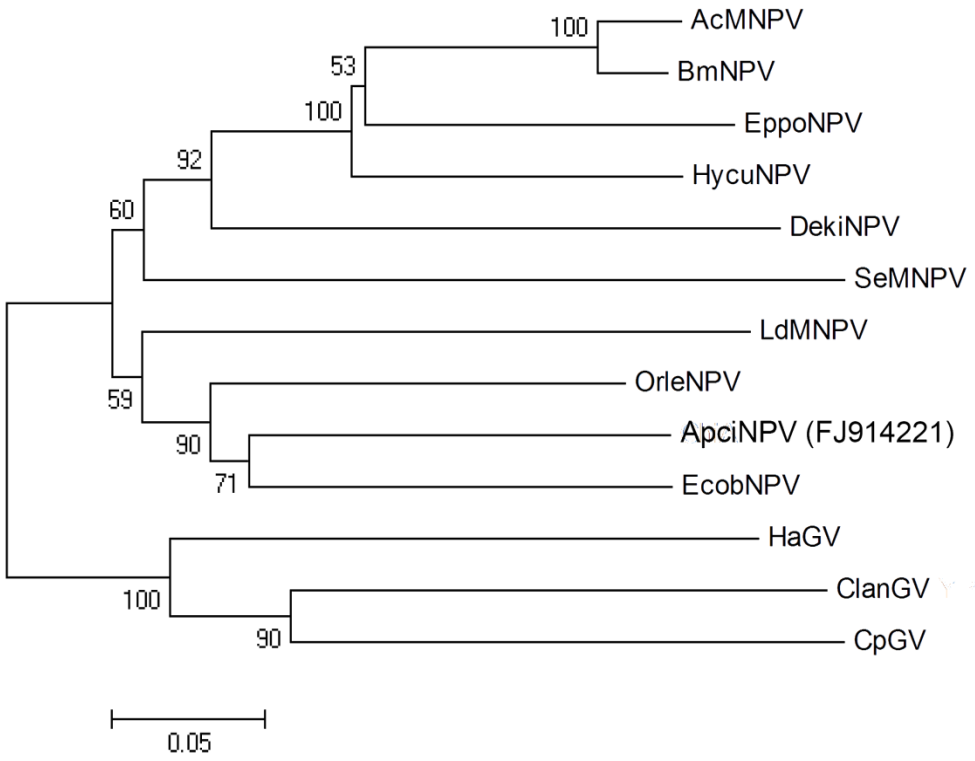

Figure 2. Constructed phylogenetic tree of chitinases from baculovirus using the neighbor-joining method. The chitinases come from ApciNPV (FJ914221), AcMNPV (NP_054156), BmNPV (NP_047523), EppoNPV (NP_203279), HycuNPV (YP_473218), DekiNPV (AFP66961), SeMNPV (NP_046280), LdMNPV (NP_047707), OrleNPV (YP_001650934), EcobNPV (YP_874243), HaGV (YP_001649087), ClanGV (YP_004376217) and CpGV (YP_148794).

at 30°C for 30 min, the reaction was terminated by 120 µL of 1 M glycine/NaOH buffer, pH10.6 for 5 min. Fluorescence was carried out using a Fluoroskan fluorimeter (Thermo Scientific Fluoroskan Asecent FL, Waltham, United States) with an excitation light at 360 nm and an emission light at 460 nm. All experiments were repeated three times.

Insect bioassays

Apocheima cinerarius, *Spodoptera*. *exigua*, *Hyphantria cunea*, *Helicoverp armigera* and *Lymantria dispar* were provided by the Research Centre of Forest Insect Virus, Chinese Academy of Forestry. Larvae were fed on artificial diet and reared at 26±°C, under a photoperiod of 14 h light/10 h dark, with 60-70% relative humidity. The insecticidal activity assay for determination of the median lethal concentrations (LC_{50}) was performed as previously described (Rajamohan et al., 1996) and partly modified. Briefly, 100 µL volume of purified ApciNPV chitinase protein solution in the concentrations of 50, 100, 200, 400 and 800 ng dissolved in elution buffer D were added to the surface of artificial diet in each 2 cm^2 well (diet surface area). Control diet was mixed with elution buffer D. The experiment was repeated three times with 20 third instar larvae (3 per well) per concentration. Starting from the fifth day after feeding with diet containing ApciNPV chitinase, the number of larvae was recorded until death or pupation. LC_{50} values were estimated by Probit analysis on the SPSS 19.0 statistical software (Finney, 1971).

Structural modeling of chitinase from ApciNPV

The homology modeling of ApciNPV chitinase was constructed by the SWISS-MODEL server (http://swissmodel.expasy.org/), and structure template was the chitinase of *S. marcescens* (PDB code: 1CTN) (Perrakis et al., 1994).

RESULTS

Analysis of chitinase gene of ApciNPV

The 1581bp *orf38* gene from ApciNPV encoded a putative chitinase at amino acid level with 65% identity to AcMNPV, 62% identity to *S. marcescens* and 58% identity to *Cydia pomonella* granulovirus (CpGV). Phylogenetic analyses of baculovirus chitinases consisted of two main branches (Figure 2) which might have much alike in properties. ApciNPV chitinase showed to belong to Alphabaculovirus genus with different percentages of identity with *Ectropis oblique* nucleopolyhedrovirus (EcobNPV,YP_874243), *Orgyia leucostigma* nucleopoly-hedrovirus (OrleNPV, YP_001650934), *L. dispar* multiple nucleopolyhedrovirus (LdMNPV, NP_047707), AcMNPV (NP_054156), BmNPV

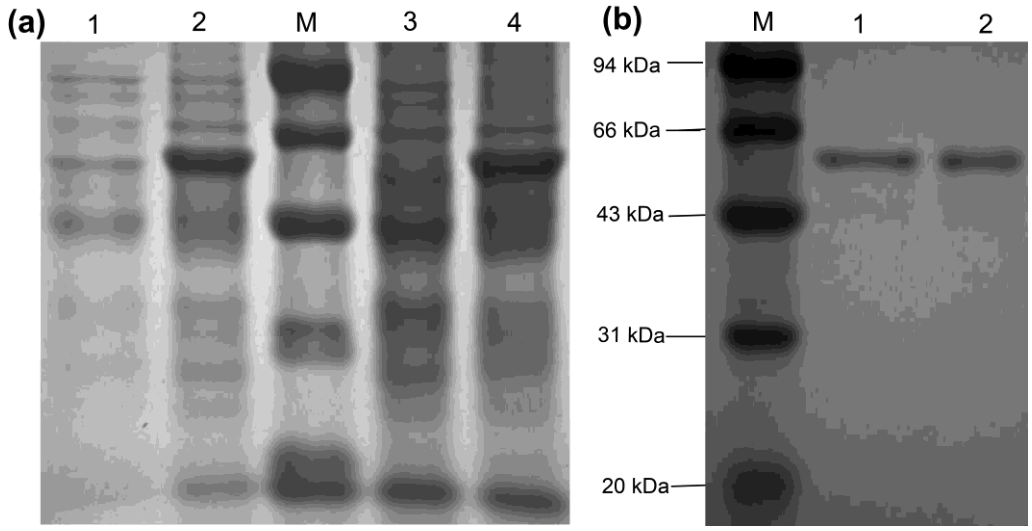

Figure 3. SDS-PAGE analysis of recombinant and purified native ApciNPV chitinase. **(a)** Expression of the recombinant ApciNPV chitinase from small-scale by IPTG induced bacterial cultures. Lane 1, the insoluble fraction of induced *E. coli* cells; lane 2, the soluble fraction; lane M, molecular mass markers (from top to down 94.0, 66.2, 43.0, 31.0, and 20.0 kDa); Lane 4, bacterial culture pellet; Lane 5, induced culture pellet using 0.2 mM IPTG at 16°C). **(b)** Large-scale IPTG-induced ApciNPV chitinase purified by Ni^{2+}-affinity chromatography. 10 µL samples containing 3 µg of protein were loaded to each lane. Lane 1 indicates the fractions washed with 20 mM Tris-HCl, 150 mM NaCl, 200 mM Imidazole, pH 7.5. Lane 2 displays the fractions eluted by 500 mM Imidazole.

(NP_047523), *Epiphyas postvittana* nucleopolyhedrovirus (EppoNPV, NP_203279), *H. cunea* nucleopolyhedrovirus (HycuNPV, YP_473218), *Dendrolimus kikuchii* nucleopolyhedrovirus (DekiNPV, AFP66961) and *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV, NP_046280).

Production and purification of native ApciNPV chitinase

The ApciNPV chitinase contained 523 amino acid residues with a calculated molecular mass of 58.2 kDa. The gene sequence was amplified according to the primers from the ApciNPV genome (GenBank accession number: FJ914221) by PCR. The PCR product was constructed into the pQE30 vector with the N-terminal 6×His-tag and then over-expressed in *E. coli* under T5 promoter transcription-translation *in vitro*. The cellular soluble fraction of recombinant protein was induced by 0.2 mM isopropyl-β-d-thiogalactoside (IPTG) at 16°C (Figure 3a, Lane 2). The recombinant His-tagged chitinase was purified using Ni^{2+}- affinity chromatography and analyzed by SDS-PAGE (Figure 3b, Lane 1 and 2).

Enzyme activity analysis of ApciNPV chitinase

The exo- and endo-chitinase activities of ApciNPV chitinase were measured by 4-MU-$(GlcNAC)_2$ and 4-MU-$(GlcNAC)_3$ fluorescent substrates, respectively. From Figure 4, both exo- and endo-chitinase activities increased linearly with the ApciNPV chitinase concentration up to 4.3-fold and 5.2-fold, respectively, over their lowest levels. It suggested that the ApciNPV chitinase produced in *E. coli* was active and exhibited its native exo- and endo-chitinolytic activities.

In vivo assays on larvae

A. cinerarius, *S. exigua*, *H. cunea*, *H. armigera* and *L. dispar* larvae were exposed to artificial diet treated with different concentrations of ApciNPV chitinase. The LC_{50} values of the larvae are shown in Table 1. The toxicities of ApciNPV chitinase response of different larvae from high to low in order were *A. cinerarius*, *S. exigua*, *H. cunea*, *H. armigera* and *L. dispar* larvae. *A. cinerarisus* larvae were the most susceptible of all tested larvae treated with the ApciNPV chitinase and LC_{50} values was 175.6 ng/cm^2 while the LC_{50} value to *L. dispar* larvae was the highest of 382.7 ng/cm^2. These results implicate the ApciNPV chitinase have an obvious insecticidal activity.

Modeling three-dimensional structure of ApciNPVchitinase

To clarify the mechanism of ApciNPV chitinase insecticidal activity, the three-dimensional structure was obtained by computer modeling (Figure 4). The model

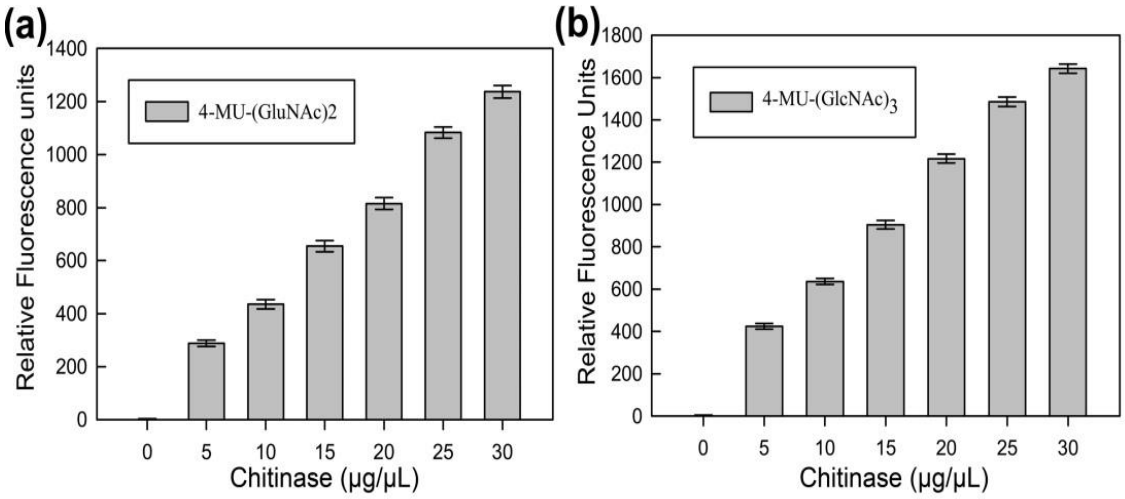

Figure 4. Enzyme activity of the recombinant ApciNPV chitinases. **(a)** The exo-chitinase activity of ApciNPV chitinase was detected using 4-MU-$(GlcNAc)_2$ substrate. **(b)** The endo-chitinase activity of ApciNPV chitinase was analyzed against 4-MU-$(GlcNAc)_3$ substrate. Results are indicated means±SE (n=3).

Table 1. Insecticidal activities of ApciNPV chitinase.

Insect species	LC_{50} (ng/cm^2)[1]	95%CL[2]	Slope
Apocheima cinerarius	175.6	73.5-543.2	2.4
Spodoptera exigua	178.5	64.2-574.5	1.8
Hyphantria cunea	279.1	87.5-628.4	3.1
Helicoverp armigera	325.4	84.3-419.7	2.8
Lymantria dispar	382.7	97.5-643.6	1.5

[1]LC_{50}, 50% lethal concentration. [2]CL=confidence limit.

was constructed using the SmChiA from *S. marcescens* which had 62% identity with chitinase from ApciNPV by SWISS-MODEL server. The ApciNPV chitinase showed three structural domains (Figure 5a), domain I was called a polycystic kidney 1(PKD1) domain producing an immunoglobulin-like fold; domain II consisted of an $(\alpha/\beta)_8$ TIM barrel, including residues 94-404 and 474-517 and domain III was composed of five antiparallel β-strands, one of which was interrupted. Furthermore, the conserved aromatic residues of ApciNPV chitinase in the hydrolysis mechanism were deduced on positions similar to SmChiA from *S. marcescens* and other chitinases (18, 19). These aromatic residues contained W28 and W31 in the immunoglobulin-like fold, W192, W205 and Y130 contributing chitin to the catalytic cleft and W127, W498, W235, Y379 and W357 binding native chitin (Figure 5b).

DISCUSSION

In this paper, the chitinase from ApciNPV was characterized. Based on amino acid sequences identity, the ApciNPV chitinase shared 65, 62 and 58% homology with the gene of AcMNPV, *S. marcescen* and CpGV, respectively. It suggests that the ApciNPV chitinase has essentially similar to the chitinase of AcMNPV and closely related to the chitinase of *S. marcescens*. The ApciNPV chitinase contained a C-terminal ER-retention sequence (HTEL) that has probably played a part in maintaining the hydrolysis in the cell at the late infection (Saville et al., 2002; Saville et al., 2004). Furthermore, compared with the known structure of SmchiA by computer model, the ApciNPV chitinase displayed a PKD1 domain and a family 18 glycohydrolase catalytic domain (Figure 5). The immunoglobulin-like fold of PKD1 domain is related to carbohydrate splitting and guiding the substrate into the catalytic groove (Bork and Doolittle, 1992; Perrakis et al., 1997). The catalytic domain possesses an open substrate-binding cleft (Uchiyama et al., 2001; Young et al., 2005). In addition, conserved tryptophan residues along the PKD1 fold and other aromatic residues in the catalytic domain have been found on the surface of SmChiA and other chitinase (Uchiyama et al., 2001; Young et al., 2005). The conserved residues of SmChiA included W69, W33, and W245, which involve in the chitin binding, and F-232 introduces the chitin chain into the catalytic pocket (Uchiyama et al., 2001). Analogously, the conserved residues from ApciNPV chitinase included

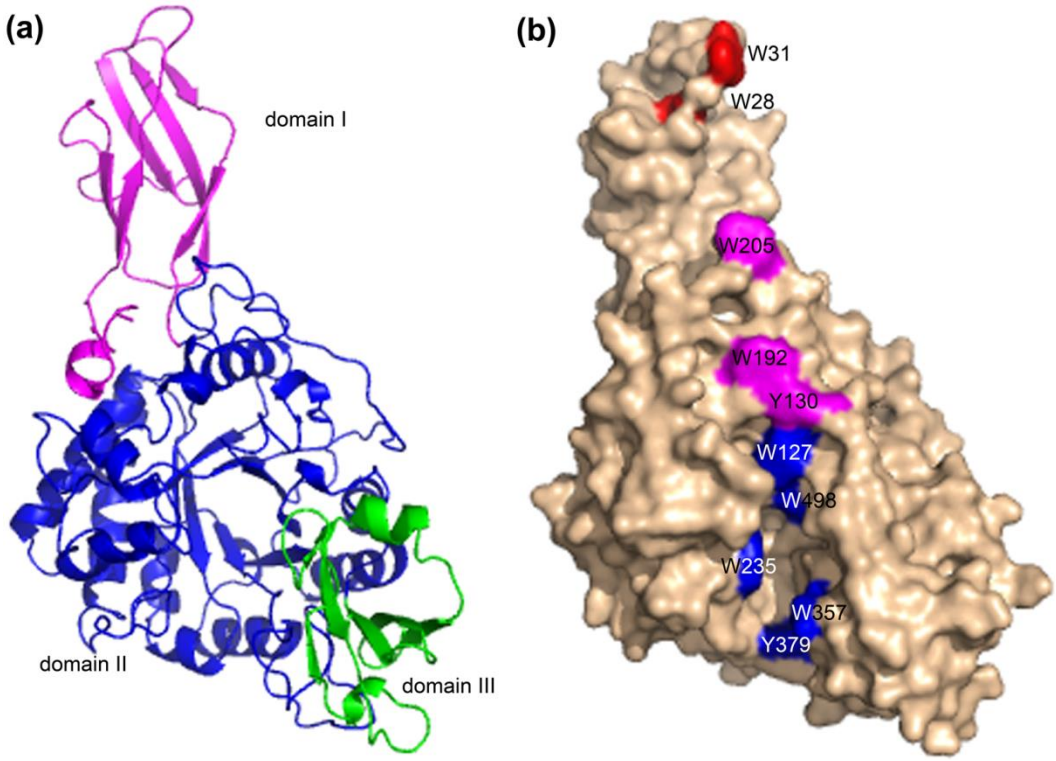

Figure 5. Modeled three-dimensional structure of ApciNPV chitinase by computer. **(a)** Ribbon drawing of ApciNPV chitinase showing the three domains in magenta (I), blue II) and green (III). **(b)** Representation of the molecular surface of ApciNPV chitinase. The conserved aromatic residues of immunoglobulin-like fold are shown red. The catalytic cleft is colored magenta. The catalytic binding-site is displayed in blue.

W28 and W31 in the corresponding position along the immunoglobulin-like fold, and W192, W205 and Y130 may also be essential for leading chitin chain into the catalytic pocket during crystalline chitin hydrolysis, and W127, Y498, W235, Y379 and W357 probably interact with GlcNAc units of chitin to forming the binding sites.

To obtain the biological activity, the truncated ApciNPV chitinase gene lacking the C-terminal ER-retention sequence (HTEL) was over-expressed in *E. coli*. The recombinant ApciNPV chitinase stored as a large amount of soluble cytosolic components (Figure 3a). The protein was efficiently purified in its native form and further identified by SDS-PAGE (Figure 3b). Furthermore, the ApciNPV chitinase was detected exo- and endo-chitinase activities with 4-MU-$(GlcNAc)_2$ and 4-MU-$(GlcNAc)_3$ substrates, respectively (Figure 4). It was in accord with previous reports stating that baculovirus chitinases have both exo- and endo-chitinases activities (Hawtin et al., 1995; Hawtin et al., 1997; Rao et al., 2004). Compared to chitinase from AcMNPV (Rao et al., 2004) and DekiNPV (Qu et al., 2011; Wang et al., 2013), the ApciNPV chitinase were higher exo- and endo-chitinase activities than the DekiNPV chitinase, yet lower exo-chitinase activity than the AcMNPV chitinase. In addition, the chitinase of ApciNPV showed an obvious insecticidal activity agianst *A. cinerarius*, *S. exigua*, *H. cunea*, *H. armigera* and *L. dispar* (Table 1). Previous studies have also indicated the role of baculovirus chitinase in pest control. For example, ChiA of AcMNPV at a sub-lethal doses (0.56 µg/g of larval body weight, LW) decreased larval body weight of *B. mori* and caused 100% mortality at a dose of 1 µg/g LW after 24 h (Rao et al., 2004). ChiA from BmNPV and AcMNPV play an important role in damaging the chitinous PM in the *B. mori* larval midgut (Rao et al., 2004; Wang et al., 2005).

The overall results indicate a chitinase from ApciNPV chitinase may offer valuable opportunities to kill insect pests under lab condition.

Conflict of Interests

The authors did not declare any conflict of interests.

ACKNOWLEDGMENTS

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REFERENCES

Cohen-Kupiec R, Chet I (1998). The molecular biology of chitin digestion. Curr. Opin. Biotechnol. 9:270-277.

Hawtin RE, Arnold K, Ayres MD, Zanotto PM, Howard SC, Gooday GA, Chappell LH, Kitts PA, King LA, Possee RD (1995). Identification and preliminary characherization of a chitinase gene in the *Autographa californica* nuclear polyhedrosis virus genome. Virol. 212(2):673-685.

Hawtin RE, Zarkowska T, Arnold K, Thomas CJ, Gooday GW, King LA, Kuzio JA, Possee RD (1997). Liquefaction of *Autographa californica* nucleopolyhedrovirus infected insects is dependent on the integrity of virus-encoded chitinase and cathepsin genes. Virol. 238(2):243-253.

Rao R, Fiandra L, Giordana B, de Eguileor M, Congiu T, Burlini N, Arciello S, Corrado G, Pennacchio F (2004). AcMNPV ChiA protein disrupts the peritrophic membrane and alters midgut physiology of *Bombyx mori* larvae. Insect Biochem. Mol. Biol. 34(11):1205-1213.

Wang F, Zhang C, Shyam Kumar V, Wu X (2005). Influences of chitinase gene deletion from BmNPV on the cell lysis and host liquefaction. Arch. Virol., 150(5):981-990.

Ohkawa T, Majima K, Maeda S (1994). A cysteine protease encoded by the baculovirus *Bombyx mori* nuclear polyhedrosis virus. J. Virol. 68(10):6619-6625.

Hom LG, Volkman LE (2000). *Autographa californica* M nucleopolyhedrovirus chiA is required for processing of V-CATH. Virol. 277(1):178-183.

Slack JM, Kuzio J, Faulkner P (1995). Characterization of v-cath, a cathepsin L-like proteinase expressed by the baculovirus *Autographa calfornica* multiple nuclear polyhedrosis virus. J. Gen. Virol. 76(5):1091-1098.

Yu ZL, Wang GC (1987). A study on the proteins of nuclear polyhedrosis virus of poplar looper, *Apocheima cinerarius*. Chinese J. Virol. 4:65-70.

Yu ZL, Wang GC (1986). Characterization of DNA isolated from nuclear polyhedrosis virus of *Apocheima cinerarius*. Chinese J. Virol. 4:85-90.

Qi YQ (2006). Experiment on ApciNPV in control over *Apocheima cinerarius* Erschoff attacking Poplars. Shaanxi For. Sci. Technol. 2:50-52.

Qu L, Wang Y, Tao J, Zhang Y (2011). Biological characteristics and multiplication of *Apocheima cinerarius* Erschoff nucleopolyhedrovirus in lab. Forest Pest and Disease, 6(30):35-37.

Wang Q, Qu L, Zhang Z, Wang Y, Zhang Y (2013). Characterization of a novel chitinase, DkChi, from *Dendrolimus kikuchii* nucleopolyhedrovirus. Arch. Virol. 158(12):2523-2530.

McCreath KJ, Gooday GW (1992). A rapid and sensitive microassay for determination of chitinolytic activity. J. Microbiol. Meth. 14:229-237.

Rajamohan F, Cotrill JA, Gould F, Dean DH (1996). Role of domain Ⅱ, loop 2 residues of *Bacillus thuringiensis* CrylAb delta-endotoxin in reversible and irreversible binding to *Manduca sexta* and *Heliothis virescens*. J. Biol. Chem. 271(5):2390-2396.

Finney DJ (1971). Probit analysis, 3rd ed. Cambridge University Press, Cambridge.

Perrakis A, Tews I, Dauter Z, Oppenheim AB, Chet I, Wilson KS, Vorgias CE (1994). Crystal structure of a bacterial chitinase at 2.3 Å resolution. Structure, 2(12):1169-1180.

Uchiyama T, Katouno F, Nikaidou N, Nonaka T, Sugiyama J, Watanabe T (2001). Roles of the exposed aromatic residues in crystalline chitin hydrolysis by chitinase A from *Serratia marcescens* 2170. J. Biol. Chem. 276(44):41343-41349.

Young VL, Simpson RM, Ward VK (2005). Characterization of an exochitinse from *Epiphyas postvittana* nucleopolyhedrovirus (family Baculoviridae). J. Gen. Virol. 86(12):3253-3261.

Saville GP, Thomas CJ, Possee RD, King LA (2002). Partial redistribution of the *Autographa californica* nucleopolyhedrovirus chitinase in virus-infected cells accompanies mutation of the carboxy-terminal KDEL ER-retention motif. J. Gen. Virol. 83(3): 685-694.

Saville GP, Patmanidi AL, Possee RD, King LA (2004). Deletion of the *Autographa californica* nucleopolyhedrovirus chitinase KDEL motif and in vitro and in vivo analysis of the modified virus. J. Gen. Virol. 85(4):821-831.

Bork P, Doolittle RF (1992). Proposed acquisition of an animal protein domain by bacteria. Proc. Natl. Acad. Sci. U.S.A. 89(19). 8990-8994.

Perrakis A, Ouzounis C, Wilson KS (1997). Evolution of immunoglobulin-like modules in chitinases: their structural flexibility and functional implications. Fold Des. 2(5):291-294.

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Full Length Research Paper

Genetic studies on common rust (*Puccinia sorghii*) of maize under Kashmir conditions

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Common rust caused by fungus *Puccinia sorghii* is among one of the worldwide spread foliar corn diseases. The disease has persisted with varying degrees of susceptibility on higher altitudes in Kashmir valley. Disease reaction studies against common rust were carried out in two crosses involving indigenously identified cytosterile source viz.,15C (A) x I-318 (R) and I-401(A) x I-318(R) for all the six basic generations with P_1, P_2 and F_1 having 30 plants each and F_2 (300 plants), BC_1 (180 plants) and BC_2 (180 plants). Analysis of variance of arc sin transformed data in the present study revealed significant differences among all the generations of both the crosses suggesting presence of sufficient variability for prevalence of diseases. Common rust screening indicated the presence of resistant genes in both the crosses which further can be exploited in the production of successful commercial hybrids by using these cytoplasmic male sterility (CMS) sources as parents to develop *Puccinia sorghii* resistant, cost effective and stable hybrids.

Key words: Maize, common rust, cytosterile source.

INTRODUCTION

Across the globe today, maize (*Zea mays* L.) is a direct staple food for millions of people and through indirect consumption as a feed crop, is an essential component of global food security. Maize is produced on nearly 100 million hectares in developing countries, with almost 70% of the total maize production in the developing world coming from low and lower middle income countries (FAOSTAT, 2010). By 2050, demand for maize will double in the developing world, while by 2025 maize is predicted to become the crop with the greatest production globally (Rosegrant *et al*., 2008).

In the state Jammu and Kashmir, maize is second most important crop after rice and is a staple food of some tribal areas such as Gujar and Bakarwall (nomadic race).

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The main maize crop is generally grown as rainfed and on marginal lands particularly in hilly terrains of the Kashmir valley. In Kashmir valley the crop is cultivated on an area of 0.1 million ha area with production of 0.15 million tonnes with an average yield of 1.2 tonnes ha^{-1} (Anonymous, 2012). This low productivity is due to both biotic and abiotic stresses and among them common rust is a problem in late planted maize grown on higher altitudes of valley and therefore should receive high priority in maize breeding research. Common rust of maize caused by *Puccinia sorghi* (Schw.) caused a yield loss of 12-60% (Utpal et al., 2012) and occurs more in spring maize growing areas (Wang et al., 2014). Very little work has been carried out in Kashmir valley on common rust due to its low prevalence in plains but on higher altitudes the disease has persisted with varying degrees of susceptibility. *Puccinia sorghi* was first described by Schweinitz from plant tissue he thought to be sorghum. While the species name would suggest sorghum is a host, *P. sorghi* does not infect Sorghum spp. The uredial and telial hosts are corn, annual teosinte (*Euchlaena* (*Zea*) *Mexicana* Schrad.), and perennial teosinte (*E.* (*Zea*) *perennis* Hitch.) (Ullstrup, 1977). Common rust may cause extensive yellowing and premature desiccation of maize foliage, resulting in leaf necrosis, and complete destruction of photosynthetic areas. In extreme cases, heavy rust infestations may result in stunting, incomplete ear tip fill and pustules on ear husks, reducing marketability and yield. Common rust of maize may be controlled by partial or hypersensitive resistance. More than 20 hypersensitive resistance *(Rp)* genes have been identified against common rust in corn germplasm (Hooker, 1969).

Thus, hybrids with resilience to *P. sorghii* provide an effective way of achieving higher production and productivity levels of the crop. But considering the cost factor cytoplasmic male sterility (CMS-source) provides a sound and sustainable alternative besides adding purity to the end product. Therefore, in the present study screening for common rust was carried out in all the six generations of both the crosses viz., 15C (A) x I-318 (R) and I-401 (A) x I-318 (R) (Where A is Female MS; Ris Male fertile parent).

MATERIALS AND METHODS

The material for study was developed by attempting the crosses 15C (A) x I-318 (R) and I-401 (A) x I-318 (R) during *Kharif* (Summer season) 2010 to generate F_1 generation at High Altitude Rice Research Sub-station, Larnoo. The F_2 and backcrosses generation (BC_1 and BC_2) were developed at Winter Nursery Centre (ICAR) Hyderabad, during *Rabi* 2010-11. All the six basic set of generations P_1, P_2, F_1, F_2, BC_1 and BC_2 and 15C (B), I-401 (B) and restorer R-line I-318 (R) of the crosses thus obtained were raised and screened for common rust.

Six generations of each cross were evaluated in randomized complete block design with three replications at the Experimental Farm of Division of Plant Breeding & Genetics, SKUAST-K, Shalimar during *Kharief* 2011. The non-segregating (P_1, P_2 and F_1) and segregating generations (F_2, BC_1 and BC_2) were raised in four and six rows with inter and intra row spacing of 60 cm and 25 cm, respectively. Screening for disease was carried out with 30 plants each of P_1, P_2 and F_1 and 300 plants of F_2, 180 plants each from BC_1 and BC_2.

Inoculum for trial was prepared from uredospores collected from corn leaves from different leaves in Kashmir valley, sealed in glass vials and stored at 21°C. Percentage of uredospores germinated was determined on 2% water agar. Uredinospore concentration was adjusted so that about 0.125 g of viable uredinospores per litre of water containing two drops of Tween-20 as wetting agent. Inoculations were made when plants were approximately at mid silk stage of growth. Rust ratios were taken 18 days after inoculation on all plants.

Assessment was done following the scale described by Zummo (1988) which is a modification and expansion of scale devised by Ullstrup (1945) as: 0=Absence of infection (no pustules); 1=slight infection (a few isolated pustules); 2=light infection (prominent pustules not so scattered); 3=moderate infection (upto 5 % of leaf area infected); 4=heavy infection (6-15% of leaf area infected); 5=very heavy infection (16% or more of leaf area infected).

The per cent disease incidence and severity were calculated at each observation as per the following formula: per cent disease incidence = number of diseased leaves / total number of leaves assessed x 100 and per cent disease severity = sum of all numerical ratings / number of leaves examined x maximum disease rating x 100. The data was arc sine transformed as recommended for data, expressed as decimal fractions or percentages as per the procedure of Steel and Torrie (1980).

RESULTS AND DISCUSSION

The observations on the evaluation of genotypes in all six generations P_1, P_2, F_1, F_2, BC_1 and BC_2 of two crosses viz., 15C (A) x I-318 (R) and I-401 (A) x I-318 (R) against *Pucciniasorghii* were recorded by calculating per cent disease incidence and per cent disease severity following (0-5) scale of Zummo (1988) respectively. The data was arc sine transformed as per Steel and Torrie (1980) and analysis of variance for the transformed data for both the crosses is presented in Table 1. Results reveal significant differences among generations of both crosses suggesting presence of sufficient variability for prevalence of diseases.

Significant critical difference of 2.06 and 2.27 with respect to rust incidence and significant critical difference of 1.96 and 1.58 with respect to rust severity were observed in cross 15C (A) x I-318 (R) and I-401 (A) x I-318 (R). Responses of the two crosses viz., 15C (A) x I-318 (R) and I-401 (A) x I-318 (R) to *Pucciniasorghii* in the trial (*Kharief* 2011) are shown in Figure 1.

Subsequently six generations of the two crosses were grouped into moderately resistant (MR) category for common rust based on the severity of disease. The crosses15C (A) x I-318 (R) and I-401 (A) x I-318 (R) were moderately resistant (MR) to common rust with mean disease severity ranging from (9.44-16.52) in 15C (A) x I-318 (R) and (9.42-17.64) in I-401 (A) x I-318 (R) cross as revealed in Table 2.

Analysis of variance detected significant differences between generations of both crosses indicating sufficient

Table 1. Analysis of variance of arc-sine transformed generation means for reaction to common rust (*Puccinia sorghii*) in two crosses I-15(A) x I-318(R) and I-401(A) x I-318(R) of maize.

Cross : I-15C(A) x I-318(R) [Rust incidence]						**Cross : I-401(A) x I-318(R) [Rust incidence]**					
S.V	**D.F**	**S.S**	**M.S**	**F**	**P**	**S.V**	**D.F**	**S.S**	**M.S**	**F**	**P**
Rep.	3	21.148	7.049	0.88	0.476	Rep.	3	6.76	2.25	0.22	0.88
Treat.	5	379.64	75.929	9.43	0.00	Treat.	5	562.02	112.40	10.82	0.000
Error	15	120.76	8.051			Error	15	155.88	10.34		
Total	23	521.56				Total	23	724			
$S.E_{(diff.)}$ = 2.006; C.D = 4.27**						$S.E_{(diff.)}$ = 2.27; C.D = 4.83**					
Cross : I-15C(A) x I-318(R) [Rust severity]						**Cross : I-401(A) x I-318(R) [Rust severity]**					
S.V	D.F	S.S	M.S	F	P	S.V	D.F	S.S	M.S	F	P
Rep.	3	0.852	0.284	0.547	0.672	Rep.	3	12.397	4.13	4.30	0.26
Treat.	5	96.506	19.301	37.18	0.000	Treat.	5	87.88	17.57	19.08	0.000
Error	15	7.792	0.519			Error	15	14.40	0.960		
Total	23	105.15				Total	23	114.677			
$S.E_{(diff.)}$ = 0.597; C.D = 1.196**						$S.E_{(diff.)}$ = 0.647; C.D = 1.588**					

** = Significant at 5% level.

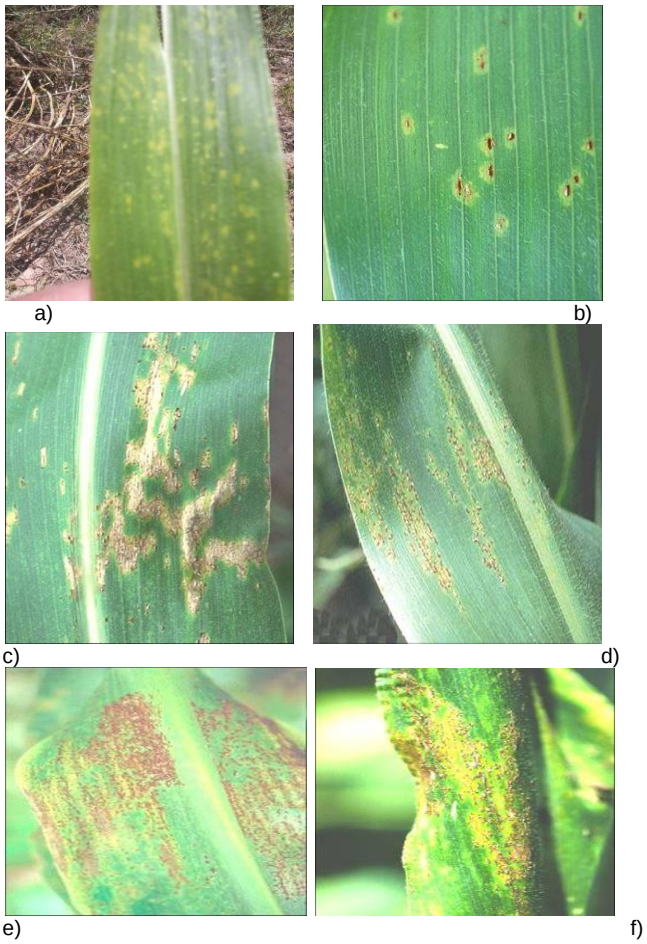

Figure 1. Common rust symptoms in two crosses viz. I-401(A) xI-318 (R) and I-15C(A) x I-318 (R). **a)** Initial symptom of leaf rust; **b)** prominent rust spores; c) ruptured rust Pustules**; d)** small amount of pustule development on leaf; **e)** uredospores of common rust; **f)** shriveled leaf after rupture of rust spores.

Table 2. Arc sine transformed mean disease incidence and severities of six generation of six generations of two crosses 15C(A) x I-318(R) and I-401(A) x I-318 (R) of maize for reaction to common rust (*Puccinia sorghii*).

Parameter		Mean disease incidence (%)	Mean disease severity (%)	Reaction
Cross 15C(A) x I-318(R)				
P_1	I-15C(A)	27.73 (31.72)	8.09 (16.52)	MR
P_2	I-318(R)	14.75 (22.52)	3.30 (9.42)	R
F_1	I-15C(A) x I-318(R)	11.06 (19.12)	2.94 (9.6)	R
F_2	I-15C(A) x I-318(R)	16.62 (24.00)	3.67 (11.03)	R
BC_1	[I-15C(A) x I-318(R)] I-15C(A)	17.54 (24.68)	4.78 (12.53)	MR
BC_2	[I-15C(A) x I-318(R)] I-318(R)	12.91 (20.94)	2.75 (9.44)	R
Cross I-401(A) x I-318(R)				
P_1	I-401(A)	31.44 (34.09)	9.20 (17.64)	MR
P_2	I-318(R)	14.75 (22.52)	3.30 (9.42)	R
F_1	I-401(A) x I-318(R)	11.07 (19.36)	5.51 (13.55)	MR
F_2	I-401(A) x I-318(R)	14.15 (22.07)	7.18 (15.49)	MR
BC_1	[I-401(A) x I-318(R)] x I-401(A)	20.32 (26.55)	7.73 (16.08)	MR
BC_2	[I-401(A) x I-318(R)] x I-318(R)	13.85 (21.64)	4.59 (12.32)	R

Source: Ullstrup (1945), Zummo (1988). Resistant (<5% of leaf area infected); MR = Moderately resistant (5.0-25.0% of leaf area infected).

variability for disease infestation but environmental factors particularly high temperature in plains might have been the limiting factor in development of maize rust. Since all the generations of both studies crosses were categorized into one group that shows moderate resistance. Little rust development in both crosses (Figure 1) reveal presence of some dominant gene which might have passed from parents. Although there was variation in disease severity between the crosses but in general all the generations of the studied crosses had fewer pustules which were significantly smaller. The percentage of pustules that had ruptured was less indicating that latent period was somewhat longer in these crosses suggesting presence of resistant genes which might be responsible for slow rusting characteristics in these crosses. Presence of slow rusting characteristics in the studied crosses reduced number and size of pustules (Figure 1), limiting secondary inoculums and thus very less plant damage was visible.

In some studies, more than 25 dominant resistance (*Rp*) genes were found to be involved in race specific resistance and organized in complex loci at chromosomes 3, 4, and 10 (Hooker, 1985; Delaney et al., 1998). Richter et al. (1995) found that within these complex loci, novel resistance specificities are generated by genetic re-assortment events, such as unequal crossing over or gene conversion. Pyramiding of multiple closely linked genes into “compound” genes has been proposed as a possible means of constructing more durable race specific resistance inherited by complex loci against common rust in maize (Hu and Hulbert, 1996). Here in this study, the results indicated presence of resistant genes in both crosses which can be further exploited in the production of successful commercial hybrids by using these CMS sources as parents to develop *P. sorghii* resistant, cost effective and stable hybrids. Also further testing of these crosses for rust resistance through molecular markers can be helpful in identifying resistant gene in commercial hybrids.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

Anonymous (2012). Directorate of Maize Research, Annual report of directorate of Maize Research, New Delhi.

Delaney DE, Webb CA, Hulbert SH (1998). A novel rust resistance gene in maize showing overdominance. Mol. Plant-Microbe Interact. 11:242-245.

Faostat (2010). Food and Agricultural Organization of the United Nations (FAO), FAO Statistical Database, from http://faostat.fao.org.

Hooker AL (1969). Widely based resistance to rust in corn. Iowa State Univ. Ext. Spec. Rpt. 64:28-34.

Hooker AL (1985). Corn and sorghum rusts. In*: The Cereal Rusts.*Roelfs AP, Bushnell WR eds. Academic Press, New York. pp. 207-236.

Hu G, Hulbert S (1996). Construction of “compound” rust resistance genes in maize. Euphytica 87:45-51.

Richter TE, Pryor TJ, Bennetzen JL, Hulbert SH (1995). New rust resistance specificities associated with recombination in the *Rp1* complex in maize. Genetics 141:373-381.

Rosegrant MW, Msangi S, Ringler C, Sulser TB, Zhu T, Cline SA (2008). International Model for Policy Analysis of Agricultural Commodities and Trade (IMPACT): Model Description. International Food Policy Research Institute,Washington, D.C.

Steel RGD, Torrie JH (1980). *Principles and procedures of Statistics*. McGraw Hill, New York, pp. 633.

Ullstrup AJ (1945). Sources of resistance to northern leaf blight. Plant Dis Rep. 47:107-108.

Ullstrup AJ (1977). Diseases of corn. In “Corn and Corn Improvement” (G. F. Sprague, ed.) Madison, Wisconsin Am. Soc. Agron. pp. 391–500.

Utpal D, Harlapur SI, Dhutraj DN, Suryawanshi AP, Badgujar SL, Jagtap GP, Kuldhar DP (2012). Spatiotemporal yield loss assessment in corn due to common rust caused by *Puccinia sorghi* Schw. Afr. J. Agric. Res. 7:5265-5269.

Wang X, Zhang Y, Xu X, Li H, Wu X, Zhang S, Li X (2014). Evaluation of maize inbred lines currently used in Chinese breeding programs for resistance to six foliar diseases. The Crop J. 2:213-222.

Zummo N (1988). Components contributing to partial resistance in maize to *Pucciniapolysora*. Plant Dis. 72: 157–160.

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Oral microflora of supragingival and subgingival biofilms in Algerian healthy adults

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The purpose of this investigation was to define the cultivable oral microflora in supragingival and subgingival plaques of Algerian healthy adults. Supragingival and subgingival plaque samples were collected from 65 Algerian caries-free, periodontally healthy subjects. Samples were taken from approximal surfaces and analysed for bacterial content after being inoculated on non-selective and selective media and incubated under different atmospheres; aerobic, capnophilic and anaerobic. The standard identification procedures by biochemical tests were used. Pearson's Chi-square χ2 test (P < 0.05, χ2-test) was used to assess the differences between the isolation frequencies. Gram negative anaerobic rods (*Porphyromonas assacharolytica, Porphyromonas gingivalis, Prevotella melaninogenica*, *Prevotella intermedia*, *Prevotella buccae*, *Fusobacterium mortiferum, Bacteroides ureolyticus*, *Bacteroides ovatus*, *Bacteroides eggertii*, *Capnocytophaga* sp. and *Aggregatibacter actinomycetemcomitans*) more often were detected in subgingival plaque than supragingival plaque (p<0.05, χ2-test). However, streptococci and *Actinomyces naeslundii* were isolated more frequently from supragingival plaque. (p<0.05, χ2-test). Facultative anaerobic Gram positive cocci were also isolated both from supragingival and subgingival plaques in comparable proportions (P>0.05, χ2-test) with the predominance of enterococci which were isolated even from supragingival and subgingival plaques in considerable proportions. The supragingival bacterial flora in healthy adults was composed mainly of Gram positive cocci and anaerobic Gram positive rods with the predominance of Streptococci and *Actinomyces naeslundii*, respectively; whereas, anaerobic Gram negative rods and facultative anaerobic Gram positive cocci were the predominant bacteria in subgingival plaque.

Keys words: Oral microflora, dental biofilm, supragingival plaque, subgingival plaque, cultivable bacteria, healthy adults.

INTRODUCTION

The commensal human microbiome is estimated to outnumber the amount of human body cells by a factor of 10 (Turnbaugh et al., 2009). These complex microbial communities are normal residents of the human body and carry a broad range of functions indispensable for the well being of the host. (Wilson, 2008).

The oral cavity, like other habitats in the body, is colonized by a characteristic and complex microbiota that grows as diverse oral biofilms. However, when the balance between the microbiota and the host is lost, the disease is manifested (Zaura et al., 2009). Some of these bacteria have been implicated in oral diseases such as caries and periodontitis, which are among the most common bacterial infections in humans. In addition, some oral bacterial species have been implicated in several systemic diseases, such as bacterial endocarditis (Berbari et al., 1997), aspiration pneumonia (Scannapieco 1999), osteomyelitis in children (Dodman et al., 2000), preterm low birth weight (Offenbacher et al., 1998; Buduneli et al., 2005) and cardiovascular disease (Beck et al., 1996; Wu et al., 2000). Despite early theories focusing on identifying a single pathogen responsible for oral diseases such as dental caries, gingivitis and chronic periodontitis, it is now generally accepted that these diseases result from the concerted actions of multispecies microbial communities of the oral biofilm (Do et al., 2013). Bacterial cultural methods were previously used as the reference method for detection of oral anaerobes, this approach is still known as gold standard to identify the major putative periodontal pathogens and a large number of oral bacteria in order to study the mechanism and nature of oral colonization, or to predict treatment outcome (Jervoe-Storm et al., 2005, Verner et al., 2006, Tomazinho and Avila-Campos 2007; Atieh 2008; Kistler et al., 2013). In contrast to an earlier view that the oral microbiome consists of large numbers of uncultivated species (Paster et al., 2001), it has been recently demonstrated that the majority of oral bacterial profiles detected by 16S pyrosequencing method could be mapped to cultivated species (Griffen et al., 2012)

The impact of the oral microbial community on shifting the balance from health to disease cannot be understood without a comprehensive view of a healthy community. Unfortunately, little attention has been paid to the human oral microbiome of the healthy oral cavity, as most studies of the human oral cavity have focused on identifying bacteria that might be associated with diseases (Becker et al., 2002; Kumar et al., 2003; Diaz et al., 2006; Kilian et al., 2006; Machado de Oliveira et al., 2007; Faveri et al., 2008). In order to diagnose and treat disease at an early and reversible stage, one needs to describe the commensal microbiome associated with health (Keijser et al., 2008). Thus, understanding changes in the oral microbiome at the early stages of periodontitis and dental caries, the most prevalent chronic oral diseases, would allow diagnosis and treatment before the appearance of periodontal pockets or dental hard tissue loss (Zaura et al., 2009). Previous studies have shown significant differences in the mean proportions of subgingival species in samples from healthy and periodontitis subjects in different countries. In fact, the microbial profiles of subgingival plaque samples differed from Swedish and American subjects who exhibited periodontal health or minimal disease (Haffajee et al., 2005). Furthermore, the microbiological profile of pooled subgingival plaque sample seemed to differ significantly between periodontal patients of Caucasian and Asian ethnic origin (Kim et al., 2009). Since there are no data on the healthy oral microbiome of adult individuals in Algeria, the present investigation aimed to estimate the detailed bacterial species richness of supragingival and subgingival microflora of the healthy adult population in the west of Algeria (Tlemcen), and more specifically to compare the cultivable bacteria of supragingival and subgingival plaques in 65 caries- free and periodontally healthy subjects.

MATERIALS AND METHODS

Subjects

Sixty five (65) subjects, representing both genders, ranging in age from 18 to 35 and with no clinical signs of oral diseases were included in the study. Subjects did not suffer from severe halitosis. They were required to have no pockets with probing depth >4 mm. Subjects did not have active white spot lesions or caries on the teeth and had a full set of natural dentition or at least 24 teeth and none of them wore any removable or fixed prosthetic appliances. They had not used antibiotics for the last three months because antibiotic therapy may change the density and composition of the normal flora and it takes weeks to return to normal. Each individual signed an informed consent document. The approval of the local Ethics Committee was obtained prior to the study.

Microbiological sampling

Sampling was performed in the morning before the participants ate breakfast; each subject was asked to refrain from eating or drinking and tooth cleaning for 12 h before sampling. Approximal supragingival plaque was selected to be sampled; this site was selected regarding its protection from cleaning and oral removal forces which enable the best development of dental biofilm at these stagnant sites.

The area to be sampled was isolated and kept dry with cotton rolls. Approximal supragingival plaque was taken with a sterile cotton swab (Citolabo, France) between the central and lateral incisor, between the premolars, and between 1st and 2nd molars. On the other side, the subgingival samples were taken from the gingival crevice in the same sites from where the approximal cotton swab samples were taken. The paper point (Revo-S^{TM}, Micro-Mega, France) was kept in place for 15 s and moved around the abutment.

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For each participant, both supragingival and subgingival plaque were collected and pooled separately into two sterile eppendorf tubes consisting each of 1 ml prereduced BHI (Brain Heart Infusion, pH 7.2 Oxoid, Basingstoke, UK) broth and processed for analysis immediately.

Cultivation

Plaque samples were processed for microbiological examination immediately after collection as follows: mixed on a Vortex shaker (IKA VibriFix, Staufen, Germany) for 30 s. Samples were diluted (10^{-1}-10^{-4}) in prereduced BHI broth and aliquots of 100 µl of each dilution and the corresponding undiluted suspension were plated onto non-selective and selective media. Columbia agar base (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) laked blood, was used to isolate cultivable facultative and anaerobic bacteria. For selective isolation of anaerobic Gram negative rods, Kanamycin -Vancomycin Laked blood agar (KVLB; Oxoid, Basingstoke, UK) was used. Chocolate agar was used for the isolation of capnophiles, MacConkey agar (Oxoid, Basingstoke, UK) for Enterobacteria, and Chapman agar (Oxoid, Basingstoke, UK) for Staphylococci. Inoculated plates were incubated at 37°C for two to seven days in different atmospheres: aerobic atmosphere, anaerobic atmosphere using an anaerobic gas generating system composed of anaerobic jar (BD GasPack^{TM} EZ container, USA) with gas container generating sachets (Genbox anaer, Biomérieux, France), and capnophilic atmosphere (5% CO_2) using CO_2 generating pouch system (GENbag, Biomérieux, France).

Identification of bacterial strains

Each different colony type from positive cultures was subcultured for purity and identification. Results from Gram-staining and atmospheric growth requirements of each colony type were used to determine the additional biochemical tests required to identify the isolates. The standard identifications of bacteria with commercial kits (API 20A, API 20Strep, API 20E, API 20 NE, and API 20Staph) (Biomérieux, France) were used. Antibiotics sensitivity testing was also used as further additional tests. Bacterial identification was achieved using the API System Electronic Codebook Program.

Statistical analysis

Statistical analysis was performed on SPSS statistics software version 22 and the Pearson's Chi-square $\chi 2$ test was used to assess the differences between the isolation frequencies. Statistical significance was set at P-value < 0.05.

RESULTS

This study was carried out in 65 caries free and periodontally healthy adults, and 130 supragingival and subgingival plaque samples were treated during this study which was conducted from October 2013 to June 2014.

Isolated bacteria

Two hundred and forty three (243) species were isolated in 130 supragingival and subgingival samples, with an average of two bacterial species per sample, the bacterial respiratory types in supragingival and subgingival samples were distributed as follows: aerobic bacteria were isolated in 54.5% of supra-gingival samples and 25.8% of subgingival samples, anaerobic bacteria were detected in 21.2% of supragingival samples and 42% of subgingival ones, while association of aerobic and anaerobic bacteria was also indicated both in supra-gingival and subgingival samples in 24.2 and 32.25% of cases, respectively (Table 1).

Distribution of isolated bacteria in supragingival and subgingival plaque according to bacterial morphotype and respiratory type

Aerobic bacteria were isolated more frequently from supragingival plaque (67.7%) compared to in subgingival plaque (30.8%). Conversely, anaerobic bacteria were more frequent in subgingival plaque (62.3%) than supragingival plaque (26.2%) ($p< 0.05$) (Figure 1). Moreover, There was a significant difference between supragingival and subgingival biofilm in Gram-positive (aerobic and anaerobic) and Gram-negative bacteria (aerobic and anaerobic) ($p<0.05$), with a large predo-minance of Gram positive bacteria in supragingival plaque (71.5%) whereas, Gram negative bacteria were more abundant in subgingival plaque (41.5%) (Figure 2).

Aerobic bacteria

Table 2 presents the distribution of aerobic bacteria in supragingival and subgingival plaques, streptococci (55.4%) (*Streptococcus intermedius, Streptococcus constellatus, Streptococcus acidomonimus, Streptococcus agalactiae, Streptococcus mutans, Streptococcus pneumoniae, Streptococcus anginosus, Streptococcus uberis* and *Streptococcus oralis*) and *Actinomyces naeslundii* (20%) were isolated more frequently from supragingival plaque ($P< 0.05$) and were the predominant bacteria of the supragingival microflora. In addition, other Gram positive cocci (*Enterococcus faecium*, *Enterococcus avium*, *Lactococcus lactis, Aerococcus viridans, Gemella haemolysans,* and *Abiotrophia defectiva)* were also isolated both from supragingival (38.5%) and subgingival (41.5%) plaques in comparable proportions ($P>0.05$) with predominance of enterococi (*E. faecium* and *E. avuim)* which were isolated even from supragingival and subgingival plaques in considerable proportions (17% and 21.5%), respectively ($p>0.05$) (Table 2).

Anaerobic bacteria

As evident from Table 3, Gram negative anaerobic rods

Table 1. Distribution of bacterial respiratory types in supragingival and subgingival samples.

Bacterial respiratory types	Aerobic bacteria	Anaerobic bacteria	Aerobic + Anaerobic Bacteria
Supragingival samples	54,5%	21.2%	24.2%
Subgingival samples	25.8%	42%	32.25%

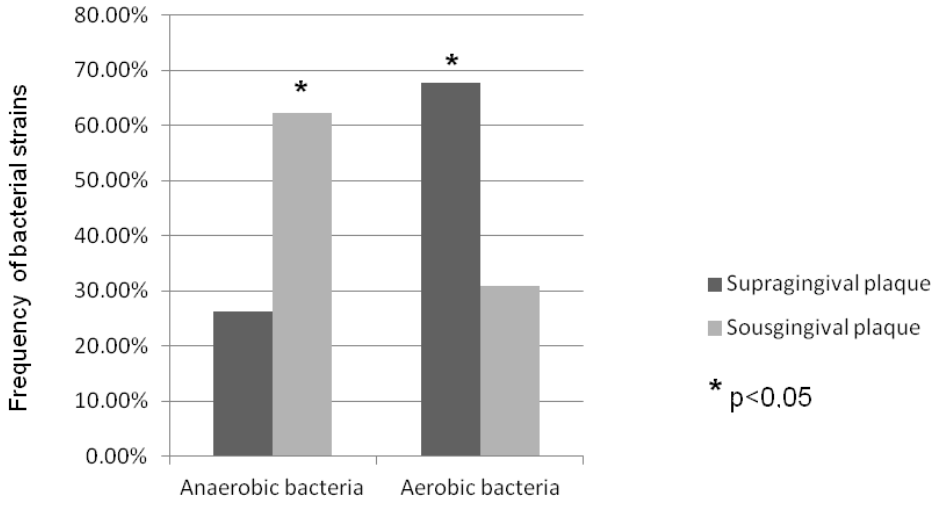

Figure 1. Anaerobi and aerobic bacteria in Supragingival and subgingival plaque of Algerian healthy adults.

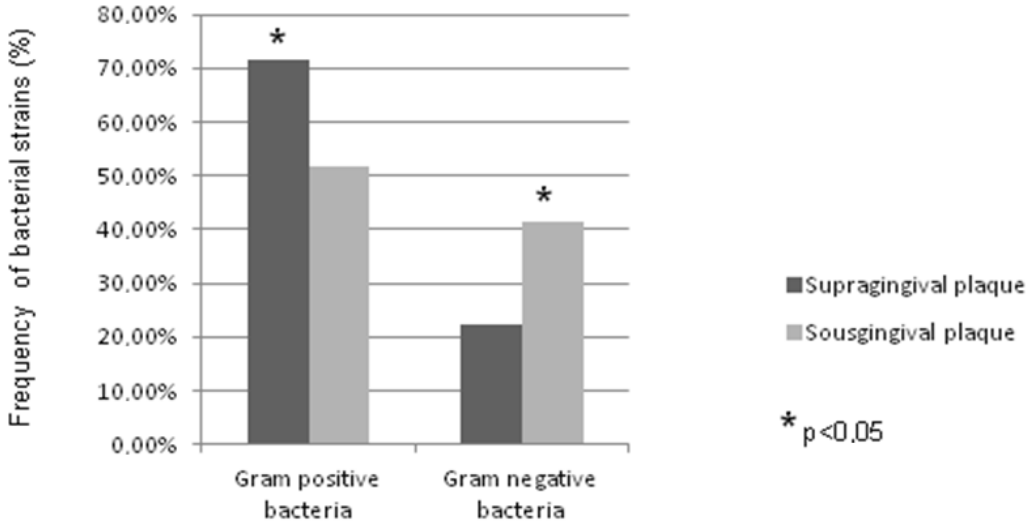

Figure 2. Gram positive and Gram negative bacteria in Supragingival and subgingival plaque of Algerian healthy.

(*Porphyromonas assacharolytica, Porphyromonas gingivalis, Prevotella melaninogenica*, *Prevotella intermedia*, *Prevotella buccae*, *Fusobacterium mortiferum, Bacteroides ureolyticus*, *Bacteroides ovatus*, *Bacteroides eggertii*, *Capnocytophaga* sp. and *Aggregatibacter actinomycetemcomitans*) were detected more often in subgingival plaque in high proportion (66.2%) ($p < 0.05$), and they were the most frequently isolated bacteria with

Table 2. Number and isolation frequencies (%) of anaerobic bacteria in supragingival and subgingival plaques of Algerian healthy subjects**.**

Bacterial species	Supragingival plaque (n= 65) Subgingival plaque (n= 65)				
	Number[a]	Frequency[b]	Number[a]	Frequency[b]	P value[c]
Anaerobic bacteria					
Peptostreptococcus sp.	0	0	2	3.07%	
Actinomyces israelii	0	0	4	6.2%	
Bfidobacterium sp.	3	4.6%	8	12.30%	p=0.009
Propionibacterium propionicum	2	3.07%	3	4.6%	
Actinomyces naeslundii	13	20%	6	9.23%	p=0.008
Lactobacillus acidophilus	2	3.07%	5	7.7%	
Lactobacillus fermentum	2	3.07%	3	4.6%	
Veillonella parvula	0	0	7	10.8%	
Anaerobic Gram negative rods	12	18.5%	43	66.2%	P=0.007
Prevotella melaninogenica	3	4.6%	3	4.6%	
Prevotella intermedia	0	0	4	6.2%	
Prevotella buccae	0	0	3	4.6%	
Porphyromonas assacharolytica	1	1.5%	3	4.6%	
Porphyromonas gingivalis	0	0	5	7.7%	
Bacteroides ureolyticus	2	3.07%	3	4.6%	
Bacteroides ovatus	0	0	2	3.07%	
Bacteroides eggertii	0	0	2	3.07%	
Fusobacterium mortiferum	0	0	2	3.07%	
Capnocytophaga sp.	6	9.23%	14	21.5%	
Aggregatibacter actinomycetemcomitans	0	0	2	3.07%	
Bacteria	34		81		

[a]Number of bacterial strains isolated from supragingival and subgingival plaques. [b]Detection frequency (%) of bacterial strains isolated from supragingival and subgingival plaques. [c]Pearson's chi-square test ($\chi 2$, $P < 0.05$) comparing the detection frequencies of bacterial strains.

predominance of *Capnocytophaga* sp. (21%) and *Prevotella* sp. (15%). *Bifidobacterium* sp. (12.3%) and *Veillonella parvula* (10.8%) were also isolated more frequently from subgingival plaque (P<0.05) (Table 3).

DISCUSSION

The findings of this study show a crucial biodiversity of the oral microflora both in supragingival and subgingival plaque of the healthy oral cavity. In supragingival samples, aerobic bacteria accounted for 54.5% of cases and 25.8% of cases in subgingival samples, while anaerobic bacteria were isolated in 21.2% of supragingival samples and 42% of subgingival ones. On the other hand, associations of aerobic and anaerobic bacteria were also observed both in supragingival (24.2%) and subgingival (32.25%) samples.

It has long been known that oral bacteria preferentially colonize different surfaces in the oral cavity as a result of specific bacterial adhesins binding to complementary specific receptors on a given oral surface (Gibbons et al., 1976; Gibbons 1989). The study of Mager et al. (2003) showed that the profiles of 40 cultivable bacterial species differed markedly on different oral environments; saliva, supragingival and subgingival plaques from healthy subjects. Such reports support the results of the present investigation that revealed a statistically significant difference in bacterial composition of supragingival and subgingival plaques of the healthy oral cavity. This difference was between aerobic bacteria which were isolated more frequently from supragingival plaque and anaerobic bacteria which were more frequent in subgingival plaque. Additionally, there was a predominance of Gram positive bacteria in supragingival plaque whereas, Gram negative bacteria were more abundant in subgingival

Table 3. Number and isolation frequencies (%) of aerobic bacteria in supragingival and subgingival plaques of Algerian healthy subjects.

Bacterial species	Supragingival plaque (n= 65)		Subgingival plaque (n= 65)		
	Number[a]	Frequency[b]	Number[a]	Frequency[b]	P value[c]
Aerobic bacteria					
Gram positive cocci except streptococci	25	38.5%	27	41.5%	P=0.089
Enterococcus sp *:*	11	17%	14	21.5%	
Enterococcus avium	1	1.5%	5	7.7%	P=0.372
Enterococcus faecium	10	15.4%	9	13.85%	
Lactococcus lactis	5	7.7%	8	12.30%	
Aerococcus viridians	5	7.7%	4	6.2%	
Gemella haemolysans	4	6.2%	0	0	
Abiotrophia defectiva	0	0	1	1.5%	
Streptococcus sp.	36	55.4%	9	13.85%	p=0.000
Streptococcus intermedius	4	6.2%	1	1.5%	
Streptococcus constellatus	2	3.07%	0	0	
Streptococcus acidomonimus	4	6.2%	3	4.6%	
Streptococcus agalactiae	5	7.7%	2	3.07%	
Streptococcus mutans	4	6.2%	1	1.5%	
Streptococcus pneumoniae	4	6.2%	0	0	
Streptococcus anginosus	5	7.7%	0	0	
Streptococcus uberis	3	4.6%	1	1.5%	
Streptococcus oralis	5	7.7%	1	1.5%	
Staphylococcus epidermidis	3	4.6%	0	0	
Staphylococcus capitis	4	6.2%	0	0	
Micrococcus sp.	3	4.6%	0	0	
Aeromonas hydrophila	2	3.07%	1	1.5%	
Moraxella sp.	2	3.07%	0	0	
Pseudomonas luteola	2	3.07%	0	0	
-Enteric rods	11	17%	3	4.6%	p=0.006
Klebsiella pneumonia	4	6.2%	1	1.5%	
Enterobacter amnigenus	3	4.6%	2	3.07%	
Serratia ficaria	4	6.2%	0	0	
Bacteria	88		40		

[a]Number of bacterial strains isolated from supragingival and subgingival plaques. [b]Detection frequency (%) of bacterial strains isolated from supragingival and subgingival plaques. [c]Pearson's Chi-square test ($\chi 2$, $P < 0.05$) comparing the detection frequencies of bacterial strains.

plaque. Rozkiewicz et al. (2006) found that Gram positive bacteria were isolated more frequently than Gram negative bacteria ($p< 0.05$) from supragingival plaques of caries free children. Oral anaerobic Gram negative rods were often defined as putative periodontal pathogens (Noiri et al., 2001). Hardly any data was given on their carriage in the healthy adults' population living in the Arab Maghreb region in particular Algeria.

However, some authors have reported the high frequency of these organisms in subgingival plaque of Algerian patients with aggressive and chronic periodontitis **(**Yacoubi et al., 2010). The present study demonstrates a high prevalence of anaerobic Gram negative rods (66.2%) in subgingival plaque of Algerian caries free and periodontally healthy adults with predominance of *Capnocytophaga sp* (21%) and *Prevotella* sp. (15%) followed by *Porphyromnas* sp. (12.3%) and *Bacteroides* sp. (10.74%). The pigmented *prevotella* species were more detected; *P. melaninogenica* (4.6%) and *P. intermedia* (6.2%). *Porphyromnas* was isolated in two species;

P. gingivalis (7.7%) and *P. assacharolytica* (4.6%). Previously, *Porphyromonas gingivalis* was not considered as belonging to the commensal oral microflora view its potential association with periodontal disease (Aas et al., 2005). Nevertheless, recent studies revealed a high prevalence of *Porphyromonas gingivalis* and other anaerobic bacteria belonging to Bacteroides phyla (*Prevotella sp, Capnocytophaga* sp., and *Bacteroides* sp.) in saliva of healthy subjects and they were less frequent in dental plaque of the same subjects (Keijser et al., 2008). The other anaerobic gram negative rods were rarely isolated in this study, *Fusobacterium mortiferum* (3.07%) and *Aggregatibacter actinomycetemcomitans* (3.07%). *Fusobacterium sp* was often detected in dental plaque of healthy subjects (Keijser et al., 2008). However, *Aggregatibacterium actinomycetemcomitans* was found associated to periodontal disease and it was very abundant in subgingival plaque of Algerian patients with aggressive periodontitis and less frequent in patients with chronic periodontitis (Yacoubi et al., 2010).

On the other hand, the results of this investigation show the importance of Gram positive cocci both in supragingival and subgingival plaque with predominance of streptococci (55.4%) in supragingival plaque. The most common species isolated were *S. oralis*, *S. anginosus*, *S. agalactiae* and they accounted each for 7.7% of cases. Several species of *Streptococcus*, including *S. sanguinis*, and *S. gordonii* were detected on the tooth surface of healthy subjects (Aas et al., 2005). In contrast, *Peptostreptococcus* sp. was rarely isolated in this study and it was detected only in subgingival plaque in low frequency (3.07%). Kumar et al. (2005) have reported the association of *Peptostreptococcus* with periodonditis due to its high carriage in these entities.

Moreover**,** our results show that *Enterococcus* sp. was very abundant in supragingival (21.5%) and subgingival plaque (17%). It has long been known that enteroccoci are the common inhabitants of the human oral cavity and they were often isolated from dental plaque (Smyth et al., 1987). However, some authors reported a high frequency of *E. faecalis* in teeth with necrotic pulp and in teeth with failing endodontic treatment (Gomes et al., 2006); this species was also associated with different forms of periradicular diseases (Rôças et al., 2004). In this study, two species of *Enterococcus* were isolated; *E. avium and E. faecium* with predominance of *E. faecium* in supragingival samples (15.4%) and subgingival ones (13.85%). Some authors' investigations aimed to inspect whether enterococci from food are able to reside in oral biofilm and showed that food-borne enterococci might not only be transient microorganisms but could also survive in the oral biofilm (Al-Ahmad et al., 2010).

Other aerobic Gram positive cocci were also detected in this study; *Lactococcus lactis* was more frequent in sub-gingival plaque (12.30%) whereas, *Gemella haemolysans* (6.2%), *Staphylococcus epidermidis* (4.6%), *Staphylococcus capitis* (6.2%)*, and Micrococcus* sp. (4.6%), were isolated only from supragingival plaque. Furthermore, *Actinomyces naeslundii* (20%) were isolated more frequently from supragingival plaque, while *Bifidobacterium* sp. (12.30%) and *Veillonella parvula* (10.8%) were more abundant in subgingival plaque. Recent findings indicated high proportions of Actinobacteria, particularly *Actinomyces* and they were higher in health and remained constant from health to periodontitis (Abusleme et al., 2013). The results of Keijser et al. (2008) showed a large abundance of streptococci and *Actinomyces* sp. in dental plaque whereas, *Veillonella parvula* was often found both in dental plaque and salivary microfolra. *Bifidobacterium* sp. and *Veillonella parvula* could frequently be isolated from subgingival and supragingival plaque and were found to be associated with periodontal disease and dental caries. (Rozkiewicz et al., 2006; Filoche et al., 2010).

Our results concurs with previous reports that showed high numbers of aerobic and facultative anaerobic Gram positive bacteria, in particular streptococci and *Actinomyces sp* with lower frequencies of anaerobes and Gram negative organisms in supragingival surfaces (Sixou et al., 2007; Do et al., 2013). In contrast, subgingival biofilm had the highest proportions of proteolytic obligate anaerobes, many of which were Gram negative anaerobes. (Sixou et al., 2007; Do et al., 2013)

Furthermore, it is interesting to note that this investigation showed a crucial biodiversity with more than 40 bacterial species of aerobic and anaerobic bacteria both in supragingival and subgingival plaques of healthy adults. Thus, enteric rods (*Klebsiella pneumonia, Enterobacter amnigenus, and Serratia ficaria)* and *Pseudomonas luteola* were also isolated in this study from supragingival plaque but in low proportion from subgingival plaque. The prevalence of oral enteric rods have been found to be in relation with oral and general health, so that an increased prevalence of oral Enterobacteriaceae carriage have been detected in patients with illnesses of varying severity compared with healthy subjects (Sedgley and Samaranayake 1994). Many authors reported that individuals in good health are able to eliminate the daily load of Gram-negative enteric rods from the oral cavity by means of innate defense mechanisms, so that bacterial counts rapidly decrease, and less than 1% of the original inoculum can be recoverable within 3-h of inoculation (Laforce et al.,1976; Mobbs et al., 1999).

In addition, other authors indicated that Gram-negative enteric rods are merely transient microorganisms within the subgingival environment both in healthy and chronic periodontitis subjects and suggested that the periodontal clinical status appeared not to be influenced by thepresence of these species (Martínez-Pabón et al., 2010). However, there was definitely a higher prevalence of Enterobacteriaceae among nail-biting individuals than

the individuals without any habit. This higher prevalence of Enterobacteriaceae among subjects with nail-biting could be due to orofecal route of transmission of Enterobacteriaceae and poor general hygiene maintenance (Baydaş et al., 2007; Reddy et al., 2013).

The crucial biodiversity of supragingival and subgingival plaques indicated in this study was confirmed by several previous reports that showed that the highest numbers and the greatest diversity of micro-organisms are found at stagnant sites within the oral cavity such as approximal surfaces which afford protection from oral removal forces (Do et al., 2013). Moreover, analysis of dental plaque in healthy adults demonstrated much more diversity than originally hypothesized (Marsh and Martin, 1999). Other authors reported that oral microbiomes of children suffering from severe dental caries are much less diverse than those of children with oral health (Kanasi et al., 2010). On the other hand, asymptomatic lesions of infected root canals displayed a higher level of biodiversity than did the symptomatic ones (Filoche et al., 2010). It has been reported also that the need for biodiversity in health may suggest that every species carries out a specific function that is required to maintain equilibrium and homeostasis within the oral cavity (Do et al., 2013). Subsequently, in health, microorganisms prevent disease progression in several ways: they can prevent the adherence of pathogens onto specific surfaces by occupying the niche preferred by a pathogen, they can actively prevent a pathogen from occupying a site, they can hinder a pathogen's abilities to multiply, and they can degrade a pathogen's virulence factors (Socransky and Haffajee, 1992).

Defining the healthy oral cavity microflora is a very important tool to understand microbial diversity and function as well as etiology of disease better, in order to diagnose diseases at an earlier and reversible stage (Zaura et al., 2009). Many studies indicated that patients with high salivary levels of potentially cariogenic bacteria such as mutants streptococci and lactobacilli, were designated as being at "high risk" for future caries, and were selected for additional clinical and therapeutic attention (Shi et al., 1998; Walsh and Tsang 2008). Such investigations share a common goal, which is to support the clinician in the diagnosis of oral diseases, providing crucial information for advanced treatment plans and therapy for "at risk" patients, and prevention strategies for healthy patients (Gibbons, 1989).

Culturing organisms remains an important tool for the detection of bacteria from dental plaque biofilm and other sites in the oral cavity in order to detect and understand pathological changes that occur within the microbial ecosystem and which may break down the ecological balance between the microbiota and the host and initiate disease within the oral cavity. This technique can detect multiple bacterial species coincidentally as it can be done by culture –independent methods, but the bacterial cultures have real advantages, that they can detect unexpected bacteria and also allow the determination of antibiotic resistance.(D'Ercole et al., 2008)

In conclusions, this investigation shows a crucial biodiversity in supragingival and subgingival plaques of the healthy oral cavity of Algerian adults. In addition, bacterial composition differed markedly in supragingival and subgingival plaques; the supragingival bacterial flora in healthy adults was composed mainly of Gram positive cocci and anaerobic Gram positive rods with the predominance of streptococci and *Actinomyces naeslundii,* respectively. Whereas, anaerobic Gram negative rods and facultative anaerobic Gram positive cocci were the predominant bacteria in subgingival plaque. Although, we confirmed previous observations of species associated with oral health, we also extend those findings, implicating additional species that will be targets for future research that could provide an important tool in understanding host–microbe interactions in health and disease. Further study of the oral microflora associated with oral health in other oral sites is also warranted and may lead to new therapeutic approaches to prevent oral diseases.

Conflict of interest

There is no conflicting interest.

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REFERENCES

Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE (2005). Defining the normal bacterial flora of the oral cavity. J. Clin. Microbiol. 43:5721-5732.

Abusleme L, Dupuy AK, Dutzan N, Silva N, Burleson JA, Strausbaugh LD, Gamonal J, Diaz PI (2013). The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. ISME. J. 7:1016-1025.

Al-Ahmad A, Maier J, Follo M, Spitzmüller B, Wittmer A, Hellwig E (2010). Food-borne enterococci Integrate Into Oral Biofilm: An In Vivo Study. Endod. 36(11):1812-1819.

Atieh MA (2008). Accuracy of real-time polymerase chain reaction versus anaerobic culture in detection of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*: a metaanalysis. J. Periodontol. 79(9):1620-1629.

Baydaş B, Uslu H, Yavuz I, Ceylan I, Dağsuyu IM (2007). Effect of a chronic nail-biting habit on the oral carriage of Enterobacteriaceae. Oral. Microbiol. Immunol. 22:1-4.

Beck J, Garcia R, Heiss G, Vokonas PS, Offenbacher S (1996). Periodontal disease and cardiovascular disease. Periodontol. 67(10):1123-1137.

Becker MR, Paster BJ, Leys EJ, Moeschberger ML, Kenyon SG, Galvin JL, Boches, SK, Dewhirst FE, Griffen AL (2002). Molecular analysis of bacterial species associated with childhood caries. J. Clin. Microbiol.

40:1001-1009.
Berbari EF, Cockerill FR, Steckelberg JM (1997). Infective endocarditis due to unusual or fastidious microorganisms. Mayo. Clin. Proc. 72(6):532-542.
Buduneli N, Baylas H, Buduneli E, Turkoglu O, Kose T, Dahlen G (2005). Periodontal infections and pre-term low birth weight: a case-control study. Clin. Periodontol. 32(2):174-181.
D'Ercole S, Catamo G, Tripodi D, Piccolomini R (2008). Comparison of culture methods and multiplex PCR for the detection of periodontopathogenic bacteria in biofilm associated with severe forms of periodontitis. New Microbiologica 31:383-391.
Diaz PI, Chalmers NI, Rickard AH, Kong C, Milburn CL, Palmer RJ, Kolenbrander PE (2006). Molecular characterization of subjectspecific oral microflora during initial colonization of enamel. Appl. Environ. Microbiol. 72:2837-2848.
Dodman T, Robson J, Pincus D (2000). Kingella kingae infections in children. J. Pediatr. Child. Heal. 36(1):87-90.
Do T, Devine D, Marsh PD (2013). Oral biofilms: molecular analysis, challenges, and future prospects in dental diagnostics. Clin. Cosmet. Invest. Dent. 5:11-19.
Faveri M, Mayer MPA, Feres M, de Figueiredo LC, Dewhirst FE, Paster BJ (2008). Microbiological diversity of generalized aggressive periodontitis by 16S rRNA clonal analysis. Oral Microbiol. Immunol. 23:112–118.
Filoche S, Wong L, Sissons CH (2010). Oral biofilms: emerging concepts in microbial ecology. J. Dent. Res. 89:8-18.
Gibbons RJ, Spinell DM, Skobe Z (1976). Selective adherence as a determinant of the host tropisms of certain indigenous and pathogenic bacteria. Infect. Immun. 13(1):238-246.
Gibbons RJ (1989). Bacterial adhesion to oral tissues: a model for infectious diseases. J. Dent. Res. 68(5):750-760.
Gomes BPFA, Pinheiro ET, Sousa ELR, Jacinto RC, Zaia AA, Ferraz CCR, Souza-Filho FJD, Piracicaba (2006). *Enterococcus faecalis* in dental root canals detected by culture and by polymerase chain reaction analysis. Oral. Surg. Oral. Med. Oral. Pathol. Oral. Radiol. Endod. 102:247-253.
Griffen AL, Beall CJ, Campbell JH, Firestone ND, Kumar PS, Yang ZK, Podar M, Leys EJ (2012). Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. ISME. J. 6:1176-1185.
Haffajee AD, Japlit M, Bogren A, Kent RL Jr, Goodson JM, Socransky SS (2005). Differences in the subgingival microbiota of Swedish and USA subjects who were periodontally healthy or exhibited minimal periodontal disease. J. Clin. Periodontol. 32(1):33-39.
Jervoe-Storm PM, Koltzscher M, Fal W, Dorfler A, Jepsen S (2005). Comparison of culture and real-time PCR for detection and quantification of five putative periodontopathogenic bacteria in subgingival plaque samples. J. Clin. Periodontol. 32:778-783.
Kanasi E, Dewhirst FE, Chalmers NI, Kent R, Moore A, Hughes CV (2010). Clonal analysis of the microbiota of severe early childhood caries. Caries. Res. 44(5):485-497.
Keijser BJF, Zaura E, Huse SM, Van der Vossen JMBM, Schuren FHJ, Montijn RC, ten Cate JM , Crielaard W (2008). Pyrosequencing analysis of the oral microflora of healthy adults. J. Dent. Res. 87(11):1016-1020.
Kilian M, Frandsen EVG, Haubek H, Poulsen K (2006). The etiology of periodontal disease revisited by population genetic analysis. Periodontol. 42:158-179.
Kim TS, Kang NW, Lee S, Eickholz P, Pretzl B, Kim C (2009). Differences in subgingival microflora of Korean and German periodontal patients. Arch. Oral. Biol. 54(3):223-229.
Kistler OJ, Booth V, Bradshaw DJ, Wade WG (2013). Bacterial Community Development in Experimental Gingivitis. Open Access, PLoS ONE 8(8):e71227.
Kumar PS, Griffen AL, Barton JA, Paster BJ, Moeschberger ML, Leys EJ (2003). New bacterial species associated with chronic periodontitis. J. Dent. Res. 82:338-344.
Kumar PS, Griffen AL, Moeschberger ML, Leys EJ (2005). Identification of candidate periodontal pathogens and beneficial species by quantitative 16S clonal analysis. J. Clin. Microbiol. 43(8):3944-55.
Laforce FM, Hopkins J, Trow R, Wang WL (1976). Human oral defenses against gram-negative rods. Am. Rev. Respir. Dis. 114:929-935.
Machado de Oliveira JC, Siqueira JF, Rocas IN, Baumgartner JC, Xia T, Peixoto RS, Rosado AS (2007). Bacterial community profiles of endodontic abscesses from Brazilian and USA subjects as compared by denaturing gradient gel electrophoresis analysis. Oral Microbiol. Immunol. 22:14-18.
Mager DL, Ximenez-Fyvie LA, Haffajee AD, Socransky SS (2003). Distribution of selected bacterial species on intraoral surfaces. J. Clin. Periodontol. 30:644–654.
Marsh P, Martin M (1999). Oral microbiology. (4th ed). London, Butterworth- Heinemann.
Martínez-Pabón MC, Isaza-Guzmán DM, Mira-López NR, García-Vélez C, Tobón-Arroyave SI (2010). Screening for subgingival occurrence of gram-negative enteric rods in periodontally diseased and healthy subjects. Arch. Oral Biol. 55:728-736.
Mobbs KJ, Van Saene HK, Sunderland D, Davies PD (1999). Oropharyngeal gram-negative bacillary carriage: a survey of 120 healthy individuals. Chest.115:1570-1575.
Noiri Y, Li L, Ebisu S (2001). The localization of periodontal-disease-associated bacteria in human periodontal pockets. J. Dent. Res. 80(10):1930-1934.
Offenbacher S, Jared HL, O'Reilly PG, Wells SR., Salvi GE, Lawrence HP (1998). Potential pathogenic mechanisms of periodontitis associated pregnancy complications. Ann. Periodontol. 3(1):233-250.
Paster BJ, Boches SK, Galvin JL (2001). Bacterial diversity in human subgingival plaque. J. Bacteriol. 183:3770-3783.
Reddy S, Sanjai K, Kumaraswamy J, Papaiah L, Jeevan M (2013). Oral carriage of enterobacteriaceae among school children with chronic nail-biting habit. J. Oral. Maxillofac. Pathol. 17(2):163-168.
Rôças IN, Siqueira Jr JF, Santos KRN (2004). Association of *Enterococcus faecalis* with different forms of periradicular diseases. J. Endod. 30:315-20.
Rozkiewicz D, Daniluk T, Zaremba ML, Cylwik-Rokicka D, Luczaj-Cepowicz E, Milewska R (2006). Bacterial composition in the supragingival plaques of children with and without dental caries. Adv. Med. Sci.51:182-186.
Scannapieco FA (1999). Role of oral bacteria in respiratory infection. Periodontol. 70(7):793-802.
Sedgley CM, Samaranayake LP (1994). Oral and oropharyngeal prevalence of *Enterobacteriaceae* in humans: a review. Oral. Pathol. Med. 23:104-113.
Shi W, Jewett A, Hume WR (1998). Rapid and quantitative detection of *Streptococcus mutans* with species specific monoclonal antibodies. Hybridoma 17(1):365-371.
Sixou M, Diouf A, Alvares D (2007). Biofilm buccal et pathologies buccodentaires. Antibiotique 9:181-188.
Smyth CJ, Matthews H, Halpenny MK, Brandis H, Colman G (1987). Biotyping, serotyping dfsband phage typing of *Streptococcus faecalis* isolated from dental plaque in the human mouth. J. Med. Microbiol. 23:45-54.
Socransky SS, Haffajee AD (1992). The bacterial etiology of destructive periodontal disease: current concepts. Periodontol. 63:322-331.
Tomazinho LF, Avila-Campos MJ (2007). Detection of *Porphyromonas gingivalis, Porphyromonas endodontalis, Prevotella intermedia*, and *Prevotella nigrescens* in chronic endodontic infection. Oral. Surg .Oral. Med. Oral. Pathol. Oral. Radiol. Endod. 103(2):285-288.
Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE (2009). A core gut microbiome in obese and lean twins. Nat. 457:480-484.
Verner C, Lemaitre P, Daniel A, Giumelli B, Lakhssassi N, Sixou M (2006). Carpegen real-time polymerase chain reaction vs. anaerobic culture for periodontal pathogens identification. Oral Microbiol. Immunol. 21(6):341-346.
Walsh L, Tsang A (2008). Chairside testing for cariogenic bacteria: Current concepts and clinical strategies. Minim. Interv. Dent. 1(2):126-149.
Wilson M (2008). Bacteriology of Humans: An Ecological Perspective. Malden, MA: Blackwell Publishing Ltd.

Wu T, Trevisan M, Genco RJ, Dorn JP, Falkner KL, Sempos CT (2000). Periodontal disease and risk of cerebrovascular disease: the first national health and nutrition examination survey and its follow-up study. Arch. Intern. Med. 160(18):2749-2755.

Yacoubi A, Bouziane D, Makhrelouf L, Bensoltane A (2010). Microbiological Study of Periodontitis in the West of Algeria. Adv. in Med. Dent. Sci. 3(3):80-85.

Zaura E, Keijser BJF, Huse SM, Crielaard W (2009). Defining the healthy "core microbiome" of oral microbial communities. BMC. Microbiol. 9:1-12.


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