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

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Full Length Research Paper

Prevalence, risk factors and major bacterial causes of bovine mastitis in and around Wolaita Sodo, Southern Ethiopia

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A cross-sectional study was conducted from November 2011 to March 2012 on lactating dairy cows to determine the overall prevalence of bovine mastitis, identify associated risk factors and isolate the predominant bacterial agents involved in causing mastitis in and around Wolaita Sodo. A total of 349 lactating cows were examined for mastitis using clinical examination and California mastitis test (CMT). Bacteriological methods were also employed to isolate the causative bacteria. An overall 29.5% (95% CI: 24.7, 34.3) prevalence of mastitis was recorded in the area of which 2.6% (95% CI: 0.9, 4.3) were clinical and 26.9% (95% CI: 22.2, 31.6) subclinical cases. About 90 bacterial isolates belonging to 6 species were identified from mastitic milk samples. The isolates based on their relative frequency of occurrence were *Staphylococcus aureus* (30%), *Streptococcus agalactiae* (17.78%), *Escherichia coli* (17.78%), *Staphylococcus epidermids* (13.3%), *Corynebacterium bovis* (12.2%) and *Streptococcus dysgalactiae* (8.9%). The prevalence of mastitis varied significantly (p<0.05) among breeds, where the highest prevalence was recorded in Jersey (60% (95% CI: 49.3, 70.7)), followed by Holstein-Zebu cross (30.8% (95% CI: 22.9, 38.7)) and Zebu (10.8% (95% CI: 5.6, 16.0)). It was also appreciated that lactation stage and parity significantly (p<0.05) influenced the occurrence of mastitis. Animals at early stage of lactation were severely affected with mastitis than at end and mid lactation stages. The prevalence of mastitis was found to have a direct relation with parity, as the occurrence of mastitis increased with parity. Inadequate sanitation of dairy environment and lack of proper attention to health of mammary gland were important factors (P<0.05) contributing to the prevalence of mastitis. Generally, the study showed that mastitis is an important problem and a serious threat for dairy industry in the study area. Therefore, appropriate control measures targeting the specific causative agents should be in place to reduce the impact of the disease. The farmers should also be aware of the impact of the disease and practice hygienic milking, culling of chronic mastitis carriers and treating of clinically infected cows.

Key words: Bacterial isolates, bovine mastitis, lactating cow, prevalence, risk factors, Wolaita Sodo.

INTRODUCTION

Ethiopia, located in tropical region, is one of the most populous countries in Africa, having an estimated population of more than 80 million. The country is very much dependent on agriculture. Livestock represent a major national resource and form an integral part of the agricultural production system. Ethiopia has the largest cattle population in Africa with an estimated population of 49.3 million. Cow represents the biggest portion of cattle population of the country, around 42% of the total cattle heads are milking cows (CSA, 2008). However, milk

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production often does not satisfy the country's requirements due to a multitude of factors. Mastitis is among the various factors contributing to reduced milk production (Biffa et al., 2005).

Mastitis is an inflammation of the mammary gland that can be caused by physical or chemical agents but the majority of the causes are infectious and usually caused by bacteria (Quinn et al., 2002; Radostits et al., 2007). Over 140 different microorganisms have been isolated from bovine intramammary infection, but the majority of infections are caused by *Staphylococci*, *Streptococci* and *Enterobacteriacae* (Bradley, 2002; Radostits et al., 2007). Mastitis is the most important and expensive disease of dairy industry. It results in severe economic losses from reduced milk production, treatment cost, increased labour, milk withheld following treatment and premature culling (Miller et al., 1993).

In view of the degree of inflammation, mastitis can be classified as clinical and sub clinical types (Philpot and Nickerson, 1991). Clinical mastitis includes gross abnormality in milk, physical abnormalities of udder and abnormality of cow with systemic involvement. Sub clinical mastitis is characterized by absence of gross lesion and an increase in number of somatic cell in the milk. It is more prevalent than clinical mastitis and with long duration, reduced production and affect quality of milk produced (Philpot and Nickerson, 1991; Radostits et al., 2007). According to Radostits et al. (2007), the diagnosis of bovine mastitis is performed by clinical examination (inspection and palpation) for clinical forms of mastitis, screening (CMT) test for subclinical forms of mastitis and bacterial isolation for confirmatory diagnosis.

In Ethiopia, mastitis has long been known (Biffa et al. 2005; Tamirat, 2007; Almaw et al., 2009; Bitew et al., 2010), however, the information on the magnitude, risk factors and causative agent of the disease is inadequate. Such information is important when designing appropriate strategies that would help to reduce its prevalence and effects (Biffa et al., 2005). Most studies in Ethiopia were carried out in Addis Ababa and its surroundings, which is not representative of other regions of the country (Almaw et al., 2009). In southern regional state, mastitis is not well considered. There is very few published material on the current status of mastitis in and around Wolaita Sodo (Tamirat, 2007). Hence this study was initiated with the objectives of determining the prevalence of bovine mastitis at Wolaita Sodo and its surroundings, assessing the associated risk factors and isolating the frequent bacterial causes.

MATERIALS AND METHODS

Study area

The study was conducted in and around Wolaita Sodo, Southern Nation Nationalities People Regional State, southern Ethiopia. Wolaita Sodo is located about 390 km south of Addis Ababa. The town Sodo is located at latitude of 8°50°N and longitude of 37°45°E. Topographically, the area is marked by hilly, flat, steep slopes and gorges and a number of streams and mountains. The highest mountain is Damota, 2500 m above sea level, which is located near Sodo town (Tamirat, 2007). The Altitude varies from 1100-2950 m.a.s.l. The area experiences mean annual temperature of about 20°C. The mean maximum temperature is 26.2°C and the average monthly minimum temperature is 11.4°C.The rainfall regimes over much of the area are typically bimodal with the big rainy season extending from June to September and a small rainy season occurring from February to April. The mean annual rain fall of the area ranges from 450-1446 mm with the lowest being in low land and highest in high land. The livestock population in the area is estimated to be 68,900 cattle, 1992 sheep, 382 goats, 121 horses, 131 mules, 488 donkeys and 55,191 chicken (Wolaita Zone Agricultural Office, 2011).

Study animals and management systems

The study populations were all lactating cows in and around Wolaita Sodo. Lactating dairy cattle (most of the time indigenous zebu cattle from surrounding areas of Sodo) visiting Sodo zuria veterinary clinic, Jersey from Wolaita Sodo dairy farm and Holstein-Zebu cross from the small holder dairy farm in Sodo town constitutes the study animals. Individual animal was selected randomly and tested for mastitis using CMT and clinical examinations.

The indigenous zebu found in the study area is managed under extensive system as a source of milk, meat and drought power. The dairy herd of Jersey breed at Sodo farm is managed more or less semi intensively under the supervision of trained personnel. Small holder dairy farms in the town are with Holstein-Zebu cross mainly and they are housed always and provided feed in their stall.

Study design

A cross-sectional study was conducted from November 2011 to March 2012 in Wolaita Sodo and its surroundings, to determine prevalence, associated risk factors, and to isolate causative agents of bovine mastitis. Sample size was determined according to Thrusfield (2005) at 95% confidence interval, 5% precision and with expected prevalence of 34.9% (Biffa et al., 2005). A total of 349 lactating cows; 130 Holstein-Zebu cross, 80 Jersey and 139 Zebu were sampled. Individual animals were selected using simple random sampling method.

Cows were examined clinically and tested for mastitis screening with CMT, and cases found clinically mastitic or screening positive were sampled for bacterial isolation. The risk factors considered at the study were, age, parity, lactation stage, breed and hygienic milking. Age of the cows was determined by observing their dentition characteristics and grouped into < 5 years, 5-8 years and > 8 years category. Parity was categorized into 1-2 calves, 3-6 calves and > 6 calves. Milking hygiene practice was grouped into good (If there is a practice of washing and drying udder with separate towels, milking healthy and young cows first) and poor (If washing and drying of udder with a separate towel and milking with order is not practiced). Lactation stage of the cow was also categorized into early stage lactation (1-4 months), mid lactation (> 4 – 8 months) and late lactation (above 8 months).

Milk sample collection

Procedure for collection of milk was according to Quinn et al. (2002); strict aseptic procedures were adopted when collecting milk samples in order to prevent contamination with microorganisms present on the body of animal and from the barn environment. Milk

sampling and screening were performed for each quarter. The time chosen for sample collection was before milking. Information on the cow age, parity, breed, lactation stage and milking hygiene were also collected at the time of sampling using data recording sheet.

Diagnosis of mastitis

In the present study, the lactating cow's udder and teats were clinically examined by palpation to know the abnormalities before the collection of milk samples. According to Radostits et al. (2007), quarters revealing the following abnormalities were diagnosed as clinical mastitis; observation of abnormal milk with no visible and palpable changes in quarters, observation of abnormal milk with visible and palpable changes in quarters and acute mastitis with systemic involvement. If one of the above symptoms was observed, milk was sampled directly for bacterial isolation. Animals or cases not showing either signs were tested for screening with CMT test; those positive were sampled to perform bacterial isolation. Sampling only positive quarters may underestimate the prevalence of subclinical mastitis; however, this can be taken as a limitation of this study.

Microbiological procedures

Samples from CMT positive and from clinical mastitic cows were analyzed microbiologically based on Quinn et al. (1999, 2002) as absolute diagnosis and identification of the disease is based on isolation and identification of bacteria. Culturing of the collected samples was performed after centrifugation to concentrate the organisms then it was inoculated into blood agar medium. MacConkey agar plate is streaked in parallel to detect gram negative bacteria. Edwards's media was used as selective for streptococcal organisms and for determination of hemolysis and aesculin hydrolysis. The inoculated plates were incubated aerobically for 24 to 48 h at 37°C. The result was declared as negative, if growth did not occur for 72 h of incubation.

Bacterial isolates were identified on the basis of colony characteristics, presence of haemolysis, Gram stain and biochemical tests. Coagulase test and maltos fermentation test (Quinn et al., 2002) were employed to differentiate *Staphylococcus aureus* from other staphylococcus species. Growth on MacConkey agar, aesculin hydrolysis on Edwards medium and CAMP test were also used to differentiate *Streptococcus agalactiae* from other mastitis causing streptococci.

Statistical analysis

Collected data were first entered into a Microsoft Excel spreadsheet and analyzed using Stata 11 software. Descriptive statistical analysis was used to summarize and present the data collected. The prevalence of mastitis was calculated as the number of lactating cows tested positive by CMT test or animals showing symptoms of clinical mastitis, divided by the total number of tested or clinically examined animals. The existence of association between the risk factors (age, parity, breed, lactation stage and milking hygiene) and mastitis was assessed using the Pearson Chi-square (χ^2) test. Besides, the degree of association between the risk factors and occurrence of mastitis were analyzed first with univariate logistic regression and those factors having a P-value less than 0.25 were further considered in multiple logistic regression analysis in the final model. The presence of interaction and adjustment for the confounding variables was not considered. Significant values were considered at P<0.05.

RESULTS

Prevalence of bovine mastitis

From the total of 349 lactating cows examined, 103 (29.5% (95% CI: 24.7, 34.3)) were positive for mastitis. Of these, 9 (2.6% (95% CI: 0.9, 4.3)) and 94 (26.9% (95% CI: 22.2, 31.6)) were found to be positive for clinical mastitis and subclinical mastitis, respectively. The results of bacterial cultures also revealed that 90 (87.4%) of the affected cows were with bacterial isolates and the rest 13 (12.6%) were without bacterial isolates. The study also considered mastitis at quarter level. Of 1396 quarters examined, 250 (17.9%) were found to be mastitis positive and 77 (5.5%) blind.

Risk factors

Among the five potential risk factors considered for a univariate logistic regression, all the risk factors age, parity, breed, lactation stage and milking hygiene were found significant (P<0.05) (Table 1). However, age became insignificant when tested with multivariate logistic regression (Table 2).

Animals over 8 years old were more frequently affected with the disease and those younger than 5 years were rarely affected (Table 1).The highest prevalence of mastitis was observed in Jersey (60%), followed by Holstein-Zebu cross (30.8%) and local Zebu (10.8%) (Table 1). The occurrence of mastitis was more than two times (OR (odds ratio) = 2.51) higher in Jersey than Zebu breeds. The difference among the three breeds was statistically significant (P<0.05) (Tables 1 and 2). Both parity and milking hygiene were found to be significantly (P<0.05) associated with the occurrence of mastitis. The occurrence of mastitis was higher in poor milking hygiene and lower at good milking hygiene. The highest prevalence of mastitis was observed in animals with parity of more than 6, followed by 3-6 and 1-2 parity (Tables 1 and 2). The occurrence of mastitis was about five times (OR = 4.83) more likely in animals with parity of more than six. Furthermore, mastitis prevalence was found to be higher in early lactation and lower in mid lactation stages (Table 1). Statistical analysis showed the existence of significant (P<0.05) association between the occurrence of mastitis and lactation stage (Table 2).

Generally, in this study, breed, parity, lactation stage and milking hygiene were considered as risk factors for the occurrence of mastitis and they were found significantly associated with the occurrence of mastitis (Table 2).

Bacterial isolation

Analysis of bacteriological examination of milk samples

Table 1. Univariate logistic regression analysis of risk factors for the occurrence of bovine mastitis in and around Wolaita Sodo, Southern Ethiopia.

Variable	Category	Number of cows examined	Number of cows with mastitis	Proportion of cows with mastitis (95% CI)	P-value	Odds ratio (OR) (95 % CI of OR)
Breed	Holstein-Zebu cross	130	40	30.8 (22.9-38.7)	0.000	1.66(1.26 - 2.18)
	Jersey	80	48	60.0 (49.3-70.7)		
	Zebu	139	15	10.8 (5.6-16.0)		
Age	<5 years	104	11	10.6 (4.7-16.5)	0.000	3.06 (2.14- 4.37)
	5 - 8 years	155	44	28.4 (21.3-35.5)		
	>8 years	90	48	53.3 (43.0-63.6)		
Milking hygiene	Good	159	20	12.6 (7.4-17.8)	0.000	0.19(0.11-0.32)
	Poor	190	83	43.7 (36.6-50.8)		
Parity	1-2	177	21	11.9 (7.1-16.7)	0.000	4.39 (2.93- 6.56)
	3-6	137	58	42.3 (34.0-50.6)		
	> 6	35	24	68.6 (53.2-84.0)		
Lactation stage	Early	94	62	65.9 (56.3-75.5)	0.000	0.44 (0.32-0.60)
	Mid	121	4	3.3 (0.1-6.5)		
	End	134	37	27.6 (20.0-35.2)		

Table 2. Multivariate logistic regression analysis of risk factors for the occurrence of bovine mastitis in and around Wolaita Sodo, Southern Ethiopia.

Variable	P-value	Odds ratio	(95 % CI)
Breed	0.000	2.51	(1.71-3.68)
Milking hygiene	0.000	0.14	(0.01-0.27)
Parity	0.000	4.83	(2.42-9.62)
Lactation stage	0.000	0.52	(0.36- 0.74)

Table 3. The frequency of bacteria isolated from bovine mastitis in and around Wolaita Sodo, Southern Ethiopia.

Organisms	Clinical mastitis	Sub clinical mastitis	Total	Relative frequency (%)
S. aureus	6	21	27	30.0
S. epidermids	-	12	12	13.3
Str. agalactiae	-	16	16	17.8
Str. dysgalactiae	-	8	8	8.9
C. bovis	-	11	11	12.2
E. coli	3	13	16	17.8
Total	9	81	90	100

was made to identify the main etiological agents involved in the disease. The organisms were identified on the basis of their cultural, staining characteristics and biochemical reactions. In the study period, about 6 bacterial species and 90 bacterial isolates: *S. aureus,* 27 (30.0%), *Str. agalactiae*, 16 (17.8%), *E. coli*, 16 (17.8%), *S. epidermids*, 12 (13.3%), *C. bovis*, 11 (12.2%) and *Str. dysgalactiae*, 8 (8.9%) were observed (Table 3).

DISCUSSION

The study was conducted on bovine mastitis in Wolaita Sodo and its surroundings to determine the prevalence and major risk factors associated with the disease. The result revealed an overall prevalence of 29.5% (955 CI: 24.7, 34.3) in the study area. This result agrees with the previous report by Bitew et al. (2010), who recorded an

overall prevalence of 28.2% at Bahir Dar and its environs. The current finding of the study is slightly lower than that of Nesru (1999) and Biffa et al .(2005), who reported an overall prevalence of 37.2% in the urban and peri-urban dairy farms at Addis Ababa, central Ethiopia and 34.9% in the southern Ethiopia, respectively. It is comparably very low when compared with the work of Sori et al. (2005) and Lakew et al. (2009), who reported a prevalence of 52.78% in and around Sebeta and 64.4% in Asella, respectively. Mastitis is a complex disease and the difference in results could be due to difference in management system of the farm, the breeds of cattle considered and the geographical locations of the studies.

The prevalence of clinical mastitis recorded in the present study is 2.6% (95% CI: 0.9, 4.3) and that of subclinical mastitis 26.9% (955 CI: 22.2, 31.6) which is by far higher than the occurrence of clinical cases. This finding is in line with the report of Bitew et al. (2010), who reported clinical prevalence of 3% and also subclinical cases of 25.2% at Bahir Dar and its environs. However, it is lower than that from the findings of Nesru (1999), who recorded 5% clinical and 32.2% sub clinical cases in the urban and peri-urban dairy farms at Addis Ababa, central Ethiopia. The results of both clinical and subclinical mastitis in this study are also incomparable with those previous reports in Ethiopia (Sori et al., 2005; Biffa et al., 2005; Lakew et al., 2009). In most reports including the present study, clinical mastitis is far lower than subclinical mastitis (Sori et al., 2005; Biffa et al., 2005; Almaw et al., 2008; Lakew et al., 2009; Haftu et al., 2012). This could be attributed to little attention given to subclinical mastitis, as the infected animal shows no obvious symptoms and secrets apparently normal milk and farmers, especially small holders, are not well informed about invisible loss from sub clinical mastitis. In Ethiopia, the subclinical forms of mastitis received little attention and efforts have been concentrated on the treatment of clinical cases (Almaw et al., 2008).

The quarter level mastitis prevalence (17.9%) recorded in the current study is comparable with the finding of Haftu et al. (2012). The finding of 5.5% blind teat in this work is in agreement with the previous reports (Almaw et al., 2008; Lakew et al., 2009) and higher when compared with the report of Haftu et al. (2012) and Bitew et al. (2010). The blind quarters observed in this study might be an indication of a serious mastitis problem on the farms and of the absence of culling that should have served to remove a source of mammary pathogens for the cows.

Association of mastitis occurrence with parity was evaluated and found statistically significant (P<0.05). The increased prevalence of mastitis with parity reported in the current study is comparable with the previous reports (Biffa et al., 2005; Tamirat, 2007; Mekibib et al., 2010; Haftu et al., 2012). This might be due to the increased opportunity of infection with time and the prolonged duration of infection, especially in a herd without mastitis control program (Radostits et al., 2007).

The occurrence of bovine mastitis and lactation stage was significantly (p<0.05) associated. That is, higher infection in cows in early lactation stage followed by late and medium lactation stages, that concurs with previous reports (Biffa et al., 2005; Tamirat, 2007). The early lactation stage infection might be due to the carryover of infection from dry period. In cows most new infections occur during the early part of the dry period and in the first two months of lactation (Radostits et al., 2007).

The study showed that breed significantly (P<0.05) influenced the occurrence of mastitis where Jerseys were severely affected than Holstein-Zebu cross or local Zebu. The report is comparable with findings of other studies such as Almaw et al. (2009) in Gondar town and its surroundings, Sori et al. (2005) in and around Sebeta and Junaidu et al. (2011) at Sokoto metropolis. However, mastitis occurrence among breeds might reflect the differences in management rather than a true genetic difference (Radostits et al., 2007). So, the higher susceptibility of Jerseys here might be the reflection of the farm hygiene and other management practices at Wolaita Sodo dairy farm.

Prevalence of mastitis was significantly (p<0.05) associated with milking hygienic practice. Cows at farms with poor milking hygiene standard are severely affected than those with good milking hygiene practices (Junaidu et al., 2011; Lakew et al., 2009; Sori et al., 2005). This might be due to absence of udder washing, milking of cows with common milkers' and using of common udder cloths, which could be vectors of spread especially for contagious mastitis.

In the current study, six bacterial species were isolated: *S. aureus*, *S. epidermids*, *Str. dysgalactiae*, *Str. agalactiae*, *E. coli* and *C. bovis*. This report closely agrees with the reports of Sori et al. (2005), Lakew et al. (2009) and Bitew et al. (2010). Bacterial isolates were recorded from all 9 clinically affected cows. The isolates from these clinical cases were *S. aureus* and *E. coli*; this is in agreement with Mekibib et al. (2010), who reported all the 12 clinical cases which were culture positive at Holeta town, central Ethiopia.

S. aureus was the predominant pathogen involved in constituting 30% of all bacterial isolates in the current study. This concurs with Delelesse et al. (2010) but not with Atyabi et al. (2006) or Mekibib et al. (2010) which were lower and higher, respectively than the current study. The relative high prevalence of *S. aureus* in the current study shows the absence of dry cow therapy and low culling rate of chronically infected animals practice in the study area.

Streptococcus species were also found prevalent with 27.7% share of the total isolates: *Streptococcus agalactiae* 17.8% and *Streptococcus dysgalactiae* 8.9%. This finding coincides with that of Hawari and Al-dabbas (2008), who reported 26.2% relative frequency of *Streptococcus* species in Jordan. However, the finding is higher than the

reports of Bitew et al. (2010) at Bahir Dar and its environs (13.9%) and Sori et al. (2005) in and around Sebeta (3.73%) and lower than the report of Atyabi et al. (2006) at farms around Tehran (33.54%).

E. coli is occurred in higher extent than expected by contributing17.8% of the isolates. The findings is different from the previous reports by Mekibib et al. (2010) at Holeta (4.6%) and Sori et al. (2005) in and around Sebeta (0.75%), but agrees with the report of Hawari and Al-dabbas (2008) in Jordan (15.6%). *E. coli* is an environmental contaminant and its high prevalence in the present report could be related to hygienic status practiced at the study site, particularly at Wolaita Sodo dairy farm where the milking hygiene practice is very poor.

There are also other coagulase negative bacteria like *S. epidermids* which contributes about 13.3% of the isolates which is in line with the report of Sori et al. (2005) in and around Sebeta (14.93%). *C. bovis* was also isolated with a relative frequency of 12.2%. This differs from other reports (Sori et al., 2005; Mekibib et al., 2010; Junaidu et al., 2011).

In conclusion, this study revealed considerable prevalence of mastitis with the isolation of major pathogens such as *S. aureus, Str. agalactiae* and *E. coli* in and around Wolaita Sodo. Therefore, appropriate control measures targeting the specific causative agents should be in place to reduce the impact of the disease on dairy industry of the study area. The farmers should also be aware of the impact of the disease and practice hygienic milking, culling of chronic mastitis carriers and treating of clinically infected cows.

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Review

Background information on the current status of South African HIV/AIDS hospices in the context of hygiene and quality of life: A review

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Human immunodeficiency virus (HIV) and the resulting acquired immunodeficiency syndrome (AIDS) continues to be a major epidemic that is ravaging the South African population at large. Hospices are non-governmental organisations that provide care to terminally ill patients, including those suffering from HIV/AIDS and cancer. This paper aims to describe the role of South African hospices in the context of the provision of palliative care, safe cooked meal and quality of life to the immune compromised patients. The role of facility design and the influence thereof on bio-aerosol level and proper hygienic practices are also elucidated. A domestic environment like a kitchen setting is known to be a source of microbial cross contamination. This article is a review on hospice kitchen layout, food borne pathogens, cross contamination and bio-aerosol prevalence.

Key words: Hospices, kitchen hygiene, HIV, food safety.

INTRODUCTION

South Africa is one of the countries most severely affected by HIV/AIDS. Approximately 1.2 million children younger than 17 years have lost one or both parents due to the epidemic (UNAIDS, 2006). This high prevalence of the HIV in South Africa has led to the establishment of hospices to assist in the care of terminally ill cancer and HIV/AIDS patients. A hospice is a non-governmental organisation that provides care to the terminally ill patients, either in their homes, in hospitals or in a hospice's own in-patients wards. In South Africa, unlike most other African countries, palliative care services can be found in hospital settings countrywide (Clark et al., 2007). A major advantage is the availability and affordability of a full multi-disciplinary team, which can provide services and support to other departments (IAHPC, 2003). Part of the hospice's mission is to offer palliative care without charge to anyone who requires it. The basic elements of hospice care include pain and symptom management, provision of support to the bereaving family and promoting a peaceful and dignified death (Johnson and Slaninka, 1999). At the core of a hospice's work is the concept of "palliative care" which is defined by the WHO (2000) as the active total care of patients whose disease is not responsive to curative treatment and whose goal is the achievement of the best quality of life for patients and their families. This service is provided without government funding and relies mainly on donations from families, charity organisations and fundraising campaigns. In South Africa, there are currently more than sixty Registered hospices linked to the national association- the Hospice and Palliative Care Association of South Africa (HPCA) which have been established since 1980. In South African health care, the present focus on cure led to health care professions turning away from patients for whom cure

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is no longer possible and abandonment of patients with far advanced illness to their families or compassionate care givers (Gwyther and Fiona, 2007). Some of the hospices are equipped with an in-patient unit as part of a home care programme. Sunflower hospice, which is situated in Bloemfontein in the Free State Province SA, provides palliative care for children only. However, Naledi hospice which is also situated in Bloemfontein only caters for adult patients. End-of-life care is an ethical imperative, which unfortunately has not been well presented in medical schools (MacDonald et al., 2000). One feature that distinguishes the natural history of HIV in non-industrialised countries from that described in rich industrialised societies is dealing with the consequences of progressive ill-health and immuno-suppression on the background of poverty and lack of resources. Abject poverty also influences disease presentation and quality of care. Again due to lack of financial resources in non-industrialised countries, it becomes difficult to implement a comprehensive and rational care packages. This review focuses on typical layout of hospice kitchens and the relationship between the design and distribution of bioaerosols.

TYPICAL LAYOUT OF SOUTH AFRICAN HOSPICES

Some local hospitals have set up direct links with a hospice's home-based care programmes, offering office or ward space in their facilities. This can be in the form of a 'step-down facility', which offers a similar service to hospice's in-patient care, but is attached to the hospital itself. For instance, both the Naledi and Sunflower hospices in Bloemfontein occupy under-utilised wards of local hospitals in their region. Patients are under the care of community caregivers and volunteer doctors, and their families are encouraged to learn palliative care skills during this time, including good hygiene and nutrition in order to strengthen the patients' resistance in the ongoing battle against opportunistic infections. Presently, there are more than 80 registered hospices in South Africa across all the provinces. Typical layout of hospice kitchen involves the following areas: main cooking area, dishwashing and storage area. This set-up is different from the hospital kitchen layout in which there is an allocated area for all the tasks, for example, hospital kitchen has receiving area, storage area and breakfast preparation area. Patients regard the food they are given as one of the most important factors in determining their quality of life. An inadequate healthcare service in which the facilities and quality of care may be very basic presents an obstacle to the proper treatment and care of HIV/AIDS patients. One important point to note in linking care with prevention is that in non-industrialised countries, the credibility of AIDS programmes will increasingly be judged by the quality of care they offer (WHO, 1992) .Trained staff may not be on hand to deliver even a limited basic package of care, either because they are not paid a living wage or, because HIV/AIDS is itself taking a toll on clinical staff.

SERIOUS BACTERIAL INFECTIONS AND HIV

Bacterial infections are a major source of morbidity and mortality in HIV-infected children, causing a wide spectrum of diseases which are included in the WHO and CDC staging systems (WHO, 2006). Serious bacterial infections occur more frequently in HIV-infected children than in HIV-uninfected children in resource-rich as well as resource-poor countries. *S. pneumoniae* is the most common pathogen causing invasive bacterial infections in HIV-infected children worldwide (Wilfert, 2000). The spectrum of bacteria associated with pneumonia in HIV-infected children is wide. The pathogens most commonly seen include *S. pneumoniae*, *H. influenzae* type B, *Staphylococcus aureus* and *Escherichia coli*. Other pathogens less commonly observed are *Streptococcus viridans*, *Streptococcus pyogenes*, *Moraxella catarrhalis*, *Bordetella pertussis*, *Klebsiella pneumoniae*, *Salmonella* spp., *Pseudomonas aeruginosa*, *Legionella* spp. and *Nocardia* spp.

During the pre-HAART era serious bacterial infections were the most commonly diagnosed opportunistic infections in HIV-infected children, with an event rate of 15 per 100 child years (Dankner et al., 2001). Pneumonia was also found to be the most common bacterial infection followed by bacteraemia and urinary tract infections. However, with the advent of HAART the rate of pneumonia has decreased to 2.2-3.1 per 100 child-years (Nachman et al., 2005). Recent data from cohort studies and mathematical models in the developed world suggest that treatment outcomes of HIV infected patients improve when antiretroviral therapy (ART) is initiated at CD4 thresholds of 350/µl, and perhaps even 500/µl (Kitahata et al., 2009). Acute lower respiratory tract infection (LRTI), diarrhea and bacteremia accounted for the majority of infections in 108 hospitalized HIV-infected children in Cape Town, South Africa (Westwood et al., 2000). In this study, none of the children received pneumococcal or *H. influenzae* vaccines, intravenous gamma globulin or ART

Food-borne pathogens and compromised immune system

Apart from the provision of quality health care by nurses and doctors in the hospices, there are food-handlers present whose main role is the provision of wholesome cooked or ready to eat meals to the patients on a daily basis. Food-handlers in the hospices play a very important role regarding the provision of safe well cooked, nutritionally balance meal to the patients. In other words, they form an important part of this continuum of care during which strict hygienic measures should be practiced throughout. Food-borne disease caused by microbiological hazards is an important global public health issue (WHO, 2000). One area that is often overlooked in preventative health care and HIV care in general is the importance of food safety. Indeed, during food preparation, pathogens such

as *Campylobacter*, *Salmonella*, *E. coli* and *S. aureus* may be spread from infected foods such as raw chicken to hand and food-contact surfaces in the domestic kitchen. Laboratory experiments have shown that both *Campylobacter* and *Salmonella* can be easily transferred from raw chicken products to kitchen surfaces and hands (Gorman et al., 2002). A food-borne illness is generally caused by micro-organisms consumed by eating any type of food. It is estimated that food-borne pathogens (disease-causing agents) are responsible for 76 million cases of illness, some resulting in death, in the United States alone every year (CDC, 2006). Campylobacteriosis is considered to be a greater burden in the developing world, partly because Campylobacter species-associated diarrhoea and bacteraemia occur in HIV/AIDS patients (Scott, 2003). The etiologic agents of food-borne illness are bacteria, viruses, parasites and food toxins with effects ranging from relatively minor discomfort to more serious symptoms and manifestations such as fever, diarrhoea, dehydration and even death (CDD, 2004). Diarrhoea, however, remains a prolific killer of children. The burden of diarrhoeal illness sits firmly in the developing world, both for morbidity (6-7 episodes per child per year when compared with 1 or 2 in the developed world) and mortality (Santosham et al., 1997).

Within the home and hospice setting, there can be a chain of events that result in the transmission of infection from its source to a new recipient. Certain sectors of the population are especially vulnerable after contracting a food-borne illness, that is, the elderly, pregnant women, young children and those with a compromised immune system (Mootsikapun, 2007). Meer and Misner (2000) have demonstrated that food-borne illness is associated with improper storage or reheating, food stored inappropriately and cross-contamination in the home. Within the hospice and health care settings in general, good hygiene practices including proper hand washing and food handling are essential in the reduction and prevention of the spread of infectious disease (Nkhebenyane et al., 2012). Although, good hygiene dictates that disinfectants should be used to clean food particles from surfaces, any bacteria remaining on these surfaces are not visible to the naked eye and may therefore be left behind. The significance of contaminated surfaces in relation to pathogen transmission to food is apparent in the food-processing, catering and the domestic environment like hospice kitchens. Pathogen exposure on surfaces may occur either by direct contact with contaminated objects or indirectly through airborne particles. Lack of food hygiene awareness and implementation are also contributing factors in this regard.

Comparison of domestic and small-scale kitchens in terms of infrastructure and cross-contamination potential

It is well known that the kitchen is particularly significant in the spread of infectious disease in the domestic environment due to various activities that occur in this particular setting. Many foods brought into the domestic kitchen are frequently contaminated with naturally occurring pathogenic microorganisms. The hospice kitchen has been described as the ‘front line in the battle against food-borne disease’, however, these kitchens may be inadequately designed, lacking equipment for safe food preparation and may be used for a range of non-food purposes.The food-preparation surfaces are a focal point in the kitchen. According to Nkhebenyane et al. (2011), in any domestic setting, the safety and quality of food served in a hospice depends on the kitchen design, storage conditions and food preparation practices of the food handlers. In this study, the environmental surfaces were found to be contaminated with pathogenic and non-pathogenic microorganisms e.g.*Staphylococcus* spp., *Bacillus* spp. and *Micrococcus* spp. The layout of a hospice kitchen is similar to that of a domestic setting, where the retention of bacteria on food contact surfaces increases the risk of cross-contamination of these micro-organisms to food (Figure 1).

Exposure of surfaces to pathogens may occur either by direct contact with contaminated objects or indirectly through airborne particles. Zhaoet al. (1998) found that during food handling and preparation, micro-organisms on raw foods can be transferred to various surfaces, such as cutting boards and water-tap spigots. The persistence of microorganisms, the presence and density of pathogens and the potential spread of microbial contamination from contaminated food in the household kitchen have been extensively studied and examined. Several studies have indicated that various bacteria, including *E. coli, S. aureus and Salmonella* spp., can survive on human hands, sponges or cloths, utensils and currency for hours or days after initial contact (Scott and Bloomfield, 1990; Kusumaningrum et al., 2002). Other studies have quantified the extent of bacterial survival and cross-contamination between hands and various food items and kitchen surfaces (Zhaoet al., 1998; Chen et al., 2001; Montvilleet al., 2001). It became evident that quantifying the cross-contamination risk associated with various steps in the food preparation process can provide a scientific basis for risk management efforts in both the home and in food service. Hand-washing and effective cleaning of food-preparation surfaces have been recognised as the most effective measures to prevent cross-conta-mination and reduce the transfer of micro-organisms to ready-to-eat foods in modern homes and institutional kitchens (Fendleret al., 2002).

MEASURES FOR PREVENTING CROSS-CONTAMINATION

Many cases of food poisoning originate in the domestic environment and can be associated with improper cleaning and food handling. The primary sources whereby

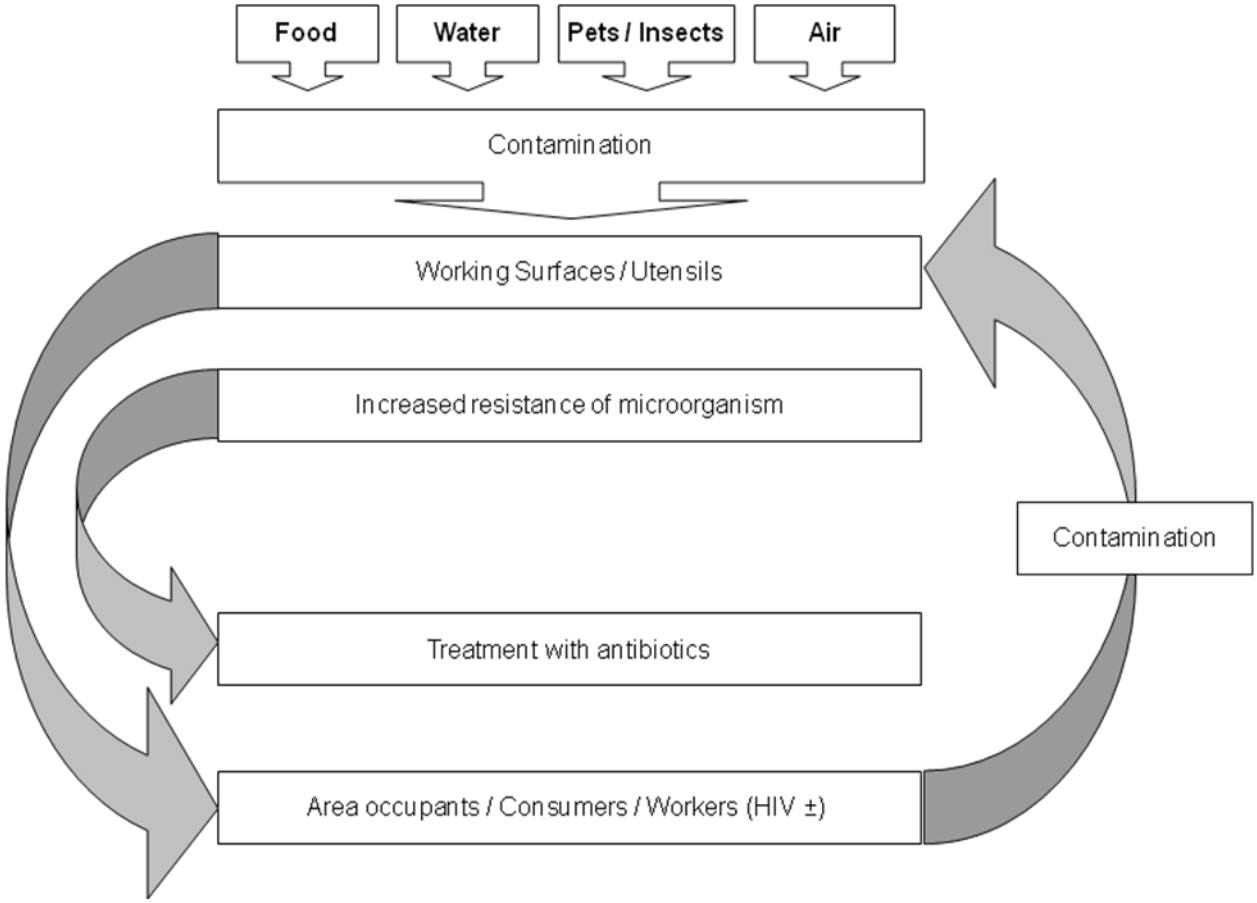

Figure 1. Chain of transmission of infection within a hospice kitchen.

pathogenic micro-organisms are introduced into the home are people, food, pets, water, insects and air (Beumeret al., 1999). Within a hospice kitchen, there is a chain of events which results in transmission of infection from its source to a new recipient. Additionally, sites where water accumulates such as sinks, toilets, waste pipes, or items such as cleaning cloths readily support microbial growth and can become primary reservoirs of infection; although species are mostly those that represent a risk to vulnerable groups. *Campylobacter* and *Salmonella* can persist on food-contact surfaces for significant period of time, which may lead to increased risks of cross-contamination between household members, ready-to-eat (RTE) foods and other food-contact surfaces (De Cesare et al., 2003). Ideally, to prevent cross-contamination in the kitchen, raw and cooked foods have to be handled separately. However, the strict separation of raw poultry, raw meat and RTE foods is not always possible in the hospice kitchen due to inadequate infrastructure.The spread of infection can be interrupted by good hygiene practices, which include good hand hygiene and the cleaning and disinfecting of surfaces. Cross-contamination of bacterial and viral pathogens in the home and in food service establishments is a major contributing factor for sporadic and epidemic food-borne illness (Knabel, 1995). Cross-contamination simulations by De Boer and Hanne (1990) demonstrated the ease with which *Campylobacter jejuni and Salmonella* are transferred from raw chicken products to chopping boards, plates and hands during food preparation. According to Kaplan (2005), the hands of food handler may also serve as a community reservoir for antimicrobial resistant strains of clinical importance, thus further emphasising the crucial role of the human hand as a vehicle for the transfer of food-borne pathogens. Simple personal hygiene coupled with soap utilisation has therefore been hailed as the most successful public health measure in the pre-disinfectant era (Greene, 2001), hence the recommendation by International Forum on Home Hygiene (IFH, 2007) that organisms be physically removed from hands and other surfaces by means of washing with soap or detergent-based cleansers, and that microbes be killed *in situ* by the application of a disinfectant or sanitizer.

However, it was demonstrated by Scott and Bloomfield (1990) that drying alone is insufficient to prevent the transfer of infectious micro-organisms between household surfaces and food handlers, and that cleaning with detergents is only a temporary measure when cloths are

kept moist. Although heat is an effective form of disinfection, Beumer et al. (1999) conceded that it may not be a possibility when it comes to large surface areas and might be unreliable in unskilled hands. In order to reduce the risk of sponges and cloths being contaminated with microorganisms, it is recommended that these items be soaked in a bleach solution or be heated for one minute in a microwave oven, or alternatively be immersed in boiling water for five minutes (IFH, 2000).

The influence of bioaerosols in a hospice setting

Exposures to bioaerosols in the occupational environment are associated with a wide range of health effects with major public health impact. According to Zuckeret al. (2000), bio-aerosols consist of all airborne particles of biological origin, that is, bacteria, fungi, fungal spores, viruses and pollen fragments, including various antigens. The transport and ultimate settling of bio-aerosol are affected by their physical properties (size, density and shape of droplets) and environmental factors which include, air current magnitude, relative humidity and temperature (Stetzenbach et al., 2004). Airborne bacteria and fungi can be the cause of a variety of infectious diseases as well as allergic and toxic effects. Healthcare facilities are complex settings, especially in developing countries, where factors such as overcrowding, improper design and ventilation can impact the growth and/or survival of microorganisms. Exposure of surfaces to pathogens may take place either by direct contact with contaminated objects or indirectly through airborne particles (Kusumaningrumet al., 2002). Pathogens are continually introduced into the home by people (who may have an infection or may be asymptomatic carriers of infectious organisms), contaminated food and domestic animals, and sometimes in water or via the air. A typical example of such a disease is tuberculosis (TB) which is caused by aerosolized *Mycobacterium tuberculosis*, which is spread from person to person through airborne particles released through a cough or sneeze by an infected person (Kumar et al., 2007).

One of the most important routes for transmission of infection is via the hands. Person-to-person and food-to-person spread via hands or surfaces, via an infected food handler or by air-borne spread can also occur. Inadequate cooking and storage of food is considered to be the main cause of food-borne infection, but poor hand and surface hygiene is also a significant contributory factor. One of the major health problems in the hospices are the episodes of diarrheal infections which are caused by a variety of bacteria, viruses and protozoa. The organisms are shed in large numbers in the faeces of an infected person. All of these organisms are transmitted via the faecal-oral route (from the faeces of an infected person or animal, to the mouth of another person). For bacterial diseases, faecal-oral routes with longer transmission cycles are also possible, such as contamination of crops or water sources with faecal material. Therefore hand hygiene is a critical component of infection control in the hospices. The following bacteria, *S. aureu*s and meticillin-resistant *S. aureus* (MRSA), *Acinetobacter* spp., *Aspergillus* spp., *Pseudomonas* spp. and *Legionella* spp. were identified as the main cause of nosocomial infection through airborne transmission in health care setting (Srinivan et al., 2002). Preventing the spread of pathogens that are transmitted by the airborne route requires the use of special air handling and ventilation systems to contain and then safely remove the infectious agent (CDC, 2005). However, these remains a challenge for the hospice kitchens since most of them rely on natural ventilation to dilute the airborne pathogens.

A South African study (Nkhebenyane et al., 2011) on the prevalence of bioaerosols in the hospice kitchens revealed the following micro biota to be mainly present: *Bacillus cereus, S. aureus, Pseudomonas* spp and coliforms. Nosocomial infections transmitted by the airborne route, especially fungal infections such as aspergillosis, have been reported by Dykewicz (2001) to be the major source of morbidity and mortality in immuno-compromised patients. Therefore, optimal hand hygiene behavior is considered the cornerstone of healthcare associated infection (HCAI) prevention (Pittet et al., 2006). According to Goodman et al. (2008), hand hygiene, together with other specific prevention measures, environmental cleaning is another essential measure to prevent the spread of some pathogens, particularly *Clostridium difficile*, vancomycin-resistant enterococci (VRE), norovirus, *Acinetobacter* spp. and methicillin resistant *S. aureus* (MRSA). Chlorhexidine is a broad spectrum antiseptic agent active against both Gram-positive and negative bacteria, and has been successfully assessed as an effective skin antiseptic since the early 1980s (Edmiston et al., 2007).Chlorhexidine, as an active antiseptic can be used directly as solution, or as an ingredient in soaps, gels or impregnated in cloths. Published studies suggest that the routine use ofchlorhexidine-gluconate (CHG) is associated with a reduction in VRE acquisition in patients in intensive care units (Climo et al., 2009). Therefore, this can also be used in the hospice by both the patients and food-handlers as an intervention strategy for improved hand hygiene and microbial load reduction.

Antimicrobial resistance and its impact on HIV treatment

Antimicrobial resistance has emerged as a major public health concern globally. For more than half a century, antibiotic drugs have made it possible to treat potentially life-threatening bacterial infections. They have turned bacterial infections into treatable conditions rather than the life-threatening compounds they once were. Schlundt (2002) identified *Campylobacter, Salmonella, Yersnia,* pathogenic *E. coli and Listeria* as the major food-borne bacterial pathogens. Infection with any one of these bacte-

rial strains, if resistant to antibiotics, will cause delays in the administration of appropriate therapy and may affect the outcome negatively (Molbak, 2005). The increasing prevalence of antibiotic resistance poses a serious threat to healthcare, and people living with HIV/AIDS are at particular risk (Manges, 2001). Furthermore, the emergence of multi-drug resistance bacteria has created a situation where there are few treatment options available for certain infections (WHO, 2002). One of the major disadvantages of antimicrobial use in animals is the potential development of antimicrobial-resistant zoonotic food-borne bacterial pathogens and the subsequent transmission thereof to humans as food contaminants. Lately, the effectiveness of many antibiotics is diminishing dramatically in the face of increasing resistance amongst various types of bacteria. Antibiotic resistance in food-borne pathogens has become a reality, although substantial qualitative and quantitative differences do exist (Teuber, 1999). A study (Nkhebenyane et al., 2012) in a hospice revealed a decrease in susceptibility for cefoxitin in approximately 80% of all *S. aureus* isolates and coliforms, while 95% of the *Staphylococcus* spp. and 93% of the *B. cereus* isolates were found to be resistant. These pathogens were isolated from the food preparation surfaces (e.g. cutting board, food handler's hands and table). *S. aureus* isolates from a tertiary hospital were reported to be 100% resistant to cefoxitin according to published results (Vysakh and Jeya, 2013). Infections caused by resistant bacteria have been shown to be more frequently associated with increased morbidity and mortality than those caused by susceptible pathogens, which poses a serious public health concern (Helmset al., 2002; Travers and Barza, 2002; Varmaet al., 2005). It is now collectively accepted that the use of antimicrobials in both animals and humans can select for resistant bacterial populations. Thus, addressing the issue of antimicrobial resistance is one of the most urgent priorities in the field of infectious disease today.

CONCLUSIONS

Hospices have been established to improve the quality of life for terminally ill patients, including those infected with HIV/AIDS. In an era of antiretroviral use as a means of reducing the viral load, food safety may relate to HIV mortality, keeping in mind the compromised immune status of the patients. Microorganisms are ubiquitous in nature, some are responsible for food-borne illnesses others posing as opportunistic pathogens- hence the importance of hygiene interventions within the hospice setting. Hospice kitchens operate on a similar basis to traditional home-based kitchens with regard to infrastructure. Although, the occupants of these settings exemplify hospital patients, this brings forth the question of whether a typical hospice kitchen will host different kinds of micro-organisms at different levels than home-based kitchens and whether the patients are contributing as a source.

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Diversity of cultivable microorganisms of *Davidia involucrate* in rhizosphere soil

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The purpose of this study was to assay the cultivable microorganisms of *Davidia involucrate* in rhizosphere soil and to analyze their diversity using colony and individual morphology and genetic profiling methods. A total of 118 strains were obtained after colony characterization and a microscopic examination, including 52 bacteria strains, 27 actinomycetes strains and 39 fungi strains. Fifty-seven (57) of these strains were randomly selected for rDNA analysis and phylogenetic identification, including 23 rhizobaceria isolates, 29 fungi isolates, and five streptomyces isolates. The selected isolates were accurately identified at the genus level with consistent results using a morphology examination and phylogenetic identification. From the study, we concluded the following: (1) *Bacillus*, *Lactobacillus*, *Azotobacter*, and *Streptomyces* are predominant microorganisms of *D. involucrate* in rhizophere soil and *Bacillus* is a dominant rhizobacteria that might have potential in host growth promotion; (2) the physiological activity of dovetree could significantly influence the microorganism biomass of the rhizophere soil; and (3) the dovetree is a highly complex rhizosphere ecosystem, and additional research on this topic should be carried out in the future.

Key words: Rhizosphere microorganism, *Davidia involucrate*, plant growth promotion, soil.

INTRODUCTION

Rhizophere microorganisms play a central role in the regulation of soil structure (Aira et al., 2010), formation of symbiosis (Filippi et al., 1995), controlling plant pathogens (Yang and Cao, 2012) and nutrient cycling (Cambardella and Eliott, 1992; Collins et al., 1992). Helman et al. (2011) summarized that among the microorganisms inhabiting the rhizosphere, several are plant growth promoting rhizobacteria (PGPR), such as genera of *Azospirillum*, *Herbaspirillum*, *Gluconacetobacter*, *Burkholderia*, *Pseudomonas*, and *Paenibacillus*. Arun et al. (2012) reported that some bacteria isolated from the rhizosphere of *Cassia occidentalis* exhibited significant growth promoting activities that could enhance root length in *Vigna radiate* and *Vigna mungo*.

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Abbreviations: PGPR, plant growth promoting rhizobacteria; **PCR,** polymerase chain reaction; **DGGE,** denaturing gradient gel electrophoresis; **SSCP,** single strand conformation polymorphism; **RFLP,** restriction fragment length polymorphisms; **T-RFLP,** terminal restriction fragment length polymorphisms; **q-PCR,** quantitative PCR; **NA,** nutrition agar; **ISSA ,** inorganic salt starch agar; **PDA ,** potato dextrose agar; **CFU,** colony forming units; **BLAST,** Basic local alignment search tool; **NCBI,** National Center of Biotechnology Information.

For assaying the genetic microorganism diversity of rhizophere soils, different culture-independent methods have been developed since Pace et al. (1985) proposed the direct cloning of environmental DNA. Most of those methods are based on the technology of polymerase chain reaction (PCR), such as denaturing gradient gel electrophoresis (DGGE) (Muyzer, 1999), single strand conformation polymorphism (SSCP) (Lee et al., 1996), restriction fragment length polymorphisms (RFLP) (Laguerre et al., 1994), terminal restriction fragment length polymorphisms (T-RFLP), (Dunbar et al., 2000), and quantitative PCR (q-PCR) (Takai and Horikoshi, 2000). Those methods have been widely used in assays of the genetic diversity of environment microbial.

Davidia involucrate is a tertiary relic species endemic to China and is reputed to be a “living fossil” in the plant kingdom. It is also an ornamental tree known as Chinese dovetree by virtue of the large pair of white bracts surrounding the small flower, which looks like a dove. In past studies, the dovetree has been well recognized for the diversity of its population, its gene expression and its distribution (Song and Bao, 2006; Wu et al., 2004; Li et al., 2002). However, the rhizophere microorganisms and their relationship with the dovetree have been infrequently studied. Therefore, in this study, we assay the cultivable microorganism biomass and diversity in the rhizosphere soil of *D. involucrate* by identifying isolations using colony and individual morphology examinations, and rDNA PCR methods. It was expected to isolate some cultivable strains that have potential to increase soil fertility or to promote host growth and that could be used as growth promotion candidate strains in further studies.

MATERIALS AND METHODS

Study site

The study site is located in the Wolong Nature Reserve of Wenchuan County, Sichuan, China (E102°56’ N30°51’ 1,718 m a.s.l.). Annual mean precipitation is 931 mm. Annual mean temperature is 8.9 ± 0.5 °C with a maximum of 29.2 ± 1.1°C (July) and a minimum of -8.5 ± 1.3°C (January). A 50-years-old natural *D. involucrate* forest was chosen to conduct this study. Canopy vegetation is dominated by *D. involucrate*, *Phoebe neuraratha*, and *Cornus controversa* with some understory shrubs (*Rhododendron violaceumv*, *Sibiraea angustata*, and *Sinarundinaria chungii*) and grass (*Polygonum viviparum*, *Cystopteris Montana*, and *Meconopsis* spp.). Soil is classified in the mountain brown soil series. The soil at a 0 to 20 cm depth had basic chemical properties of pH 5.8±0.2, 130.3±12.8 g total organic C kg^{-1} DW, 20.2±1.9 g total N kg^{-1} DW, and 3.2±0.4 g total P kg^{-1} DW.

Soil collection

Eight plots in the study site were selected and marked as GT1, GT2, GT3, GT4, and FGT1, FGT2, FGT3, FGT4. The GT plots were set as positive controls with *D. involucrate* grown. Conversely, the FGT plots were set as negative controls without *D. involucrate* grown. Soil samples were collected at depths of 0 to 20 cm after removing the surface soil (3 mm) at intervals of 1 m from the tree trunk and three soil samples were collected at each plot. The rhizosphere soil was defined as the soil attached to the root after gentle crushing and shaking of the collected roots. A total of 24 soil samples were collected. These samples were stored in freezer boxes at 4°C and transported to the laboratory within 24 h.

Rhizosphere microorganism cultivation and isolation

Rhizosphere cultivable heterotrophic microorganisms were quantified by the plate dilution method. Nutrition agar (NA) was used as selection medium for isolating bacteria (except for actinomycete), inorganic salt starch agar (ISSA) for actinomycete and potato dextrose agar (PDA, Hangzhou Microbial Reagent CO., LTD, China) for fungi, respectively. In brief, 5 g of mixed fresh soil was extracted with 45 ml sterile physiological saline solution (0.85% NaCl) in an Erlenmeyer flask by shaking the mixture for 30 min at about 150 rpm/min. immediately after shaking, the suspension experienced a series of tenfold dilution by pipetting 1 ml aliquots into 9 ml sterilized water. The final dilution was 10^9-fold, and 0.2 ml of each dilution of the series was placed onto a Petri dish. Three replicate dishes were made for each dilution. NA plates were incubated at 37°C for 48 h, ISSA and PDA plates were incubated at 28°C for 5-7 days. After incubation, the colony forming units (CFU) of each plate were counted and each typical rhizosphere microbial isolate was sub cultured on appropriate medium to obtain pure colony. Each selected typical colony was characterized according to the properties of shape, surface, color and transparency (Stainer et al., 1987), as well as an individual morphology examination through Gram’s staining. All selected strains were stored at -80°C in broth with 10% glycerol.

Colony PCR of rhizobacteria

The colony PCR method was used to amplify 16S rDNA of rhizobacteria as described by Sheu et al. (2000). Colony PCR was performed using a TIANGEN 2×Taq PCR MasterMix Kit (TIANGEN Biotech, Beijing, China) according to the manufacturer’s introduction by following universal primers: 5'-AGA GTT TGA TCC TGG CTC AG-3' (27F) and 5'-TAC GGY TAC CTT GTT ACG ACT T-3'(1492R). The colony PCR was performed in a volume of 50 µl containing 25 µl TIANGEN 2×Taq PCR MasterMix, 1 µl of 20 mmol/l 27F primer, 1 µl of 20 mmol/l 1492R primer, 23 µl sterile water, and approximately 10^3 bacterial cells. PCR conditions were performed in a DNA thermal cycler (Bio-Rad, Hercules, CA) as previous reported (Luo et al., 2012); 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1.5 min, 72°C for 10 min and 4°C for completion. The expected size of PCR products were 1500 bp. PCR amplicons were electrophoresed in 1% agarose gels and stained with Gold-View (0.005% v/v).

Genomic DNA extraction and PCR amplification of fungi

The genomic DNA of isolated fungi was extracted by a double-sedimentation method (Wu et al., 2003). PCR was performed using TIANGEN 2×Taq PCR MasterMix Kit by two universal primers: 5’-TCC GTA GGT GAA CCT GCG G-3’ (ITS_1) and 5’-TCC TCC GCT TAT TGA TAT GC-3’ (ITS_4). PCR reaction was carried out in a volume of 50 µl containing 25 µl TIANGEN 2×Taq PCR MasterMix, 1 µl of 20 mmol/l ITS_1 primer, 1 µl of 20 mmol/l ITS_4 primer, 1 µl of template DNA, 22 µl sterile water. PCR conditions were as follows: 3 min at 95°C, followed by 30 cycles of 1 min at 94°C, 1 min at 52°C, 1 min at 72°C, 72°C for 10 min, and 4°C for completion. The expected size of PCR products were 800 bp. PCR amplicons were electrophoresed in 1% agarose gels and stained with Gold-View (0.005% v/v).

Table 1. Colony forming units counting results of each soil sample.

Plot	Colony count results (CFU/g)		
	Bacteria	Actinomycetes	Fungi
GT1	$(6.25\pm2.09)\times10^6$	$(1.15\pm0.39)\times10^4$	$(2.05\pm0.34)\times10^2$
GT2	$(45.86\pm5.33)\times10^6$	$(1.13\pm0.21)\times10^4$	$(1.88\pm0.29)\times10^2$
GT3	$(4.75\pm2.15)\times10^6$	$(0.98\pm0.24)\times10^4$	$(1.69\pm0.33)\times10^2$
GT4	$(6.79\pm1.31)\times10^6$	$(1.05\pm0.18)\times10^4$	$(1.98\pm0.37)\times10^2$
FGT	$(5.84\pm1.87)\times10^6$	$(0.95\pm0.34)\times10^4$	$(1.58\pm0.49)\times10^2$

Sequencing and analysis of PCR amplicons

The complete sequencing of PCR amplicons were performed by Sangon Biotech (Shanghai, China). Closest known relatives of the isolates were determined by performing Basic Local Alignment Search Tool (BLAST) program at National Center of Biotechnology Information (NCBI) website (Altschul et al., 1990). Phylogenetic and molecular evolutionary analyses of the sequences were conducted using MEGA version 4 (Tamura et al., 2007). Multiple sequence alignments were performed and drawing of phylogenetic tree was carried out by Neighbor-Joining method (Saitou and Nei, 1987). Correction with 1000 replicates to produce bootstrap values (Felsenstein, 1985) and the phylogenetic tree was confirmed by maximum-parsimony method (Kluge and Farris, 1969) and maximum-likelihood method (Cavalli-Sforza and Edwards, 1967).

RESULTS

Colony counting results

The colonies counting results of plates after incubation are shown in Table 1. The counting results revealed that rhizobacteria was the dominant microorganisms in 24 tested soil samples. There were no significant differences in the population of rhizosphere microorganisms except for GT2. However, it is difficult to calculate the fungi colonies of tested soil samples since rare fungi colonies grew on the PDA plates, indicating that the fungi biomass was very low in each plot.

Colony and morphology characterization

A total of 118 strains were obtained after colony characterization and microscope examination, including 52 strains isolated by NA medium (labeled as NA-isolated), 27 strains isolated by ISSA medium (labeled as ISSA-isolates) and 39 strains isolated by PDA medium (labeled as PDA-isolates). The 52 NA-isolates were grouped into nine genera as shown in Table 2; genus *Bacillus* (36.53%, n=19) and *Lactobacillus* (26.92%, n=14) were dominant microorganisms in tested soil samples. It wass noticeable that there were significantly more Gram-positive strains (69.23%, n=36) than Gram-negative strains (30.78%, n=16). The 27 ISSA-isolates were identified as streptomyces genera by means of an individual morphology examination according to Taddei's reports (Taddei et al., 2006).

Colony and morphology characterization results of 39 PAD-isolates are summarized in Table 3. The isolates were divided into 11 genera as described by Wei (1979); genus *Penicillium* (15.38%, n=6), *Trichodema* (15.38%, n=6), *Aspergillus* (12.82%, n=5) and *Mucor* (12.82%, n=5) were detected as dominant fungi genera. The morphology results indicated that fungi species diversity of the tested soil samples is variable, although fungi biomass was low (only 39 isolates).

rDNA analysis and phylogenetic identification

57 isolates were randomly selected to perform rDNA analysis and phylogenetic identification, including 23 NA-isolates and 29 PDA-isolates (two isolates selected from the group which contains less than three isolates and three from those that contained more than three isolates), and 5 ISSA-isolates. Electrophoresis indicated that the expected sizes of PCR products were obtained (Figure 1). Each rDNA sequence was analyzed by BLAST program on NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) after complete sequencing. Phylogenetic trees were constructed by using the sequences of randomly selected isolates and three typical strain (ten for streptomyces genera) sequences of each closely related genus for phylogenetic identification (Figures 2, 3 and Figure 4). The phylogenetic analysis results revealed that each group of 57 randomly selected isolates was clearly separated into individual branches, which coincided with the morphology examination results as described above.

DISCUSSION

To our knowledge, it is the first time that cultivable microorganisms were assayed in *D. involucrate* rhizosphere soil. In this study, we found that the biomass of rhizobacteria in GT2 was much higher than the other plots (Table 1). According to previous studies, physiological activity of plant could influence the rhizobacteria biomass. Wang et al. (2010) found that plants are able to regulate the soil microbial community in their immediate vicinity through the secretion from *Rehmannia glutinosa* roots to the rhizosphere. Arun et al. (2012) reported that the

Table 2. Colony and morphology characterization of rhizobacteria isolates.

Group	Strains number	Gram staining	Spore	Colony characterization				Individual morphology	Genera
				Shape	Surface	Color	Transparency		
1	19	+	+	Radiciform	Rough	Milk-white	Translucent	Rod	*Bacillus*
2	3	-	-	Round	Smooth	Purple	Opaque	Rod	*Chromobacterium*
3	2	-	-	Round	Smooth	Greenyellow	Opaque	Short rod	*Vibrio*
4	2	-	-	Round	Smooth	-	Transparent	Rod	*Agrobacterium*
5	2	-	-	Round	Smooth	Milk-white	Translucent	Rod	*Photobacterium*
6	3	+	-	Round	Smooth	-	Translucent	Cocci	*Micrococcus*
7	2	-	-	Round	Smooth	Pink	Opaque	Rod	*Serratia*
8	14	+	-	Round	Smooth	Milk-white	Opaque	Rod	*Lactobacillus*
9	5	-	-	Irregular	Mucoid	-	Translucent	Rod	*Azotobacter*

+, positive; -, negative.

Table 3 Colony and morphology characterization of fungi isoltes.

Group	Strains number	Colony color	Hyphae	Septum	Spore				Genera
					Type	Shape	Size (μm)	Color	
1	3	White	Flocculence	-	Sporangiospore	Reniform	13-19	Transparent	*Pythium*
2	2	Brown	Stolon	-	Sporangiospore	Oval	4-10	Gray	*Rhizopus*
3	5	Gray	Flocculence	+	Sporangiospore	Oval	4-9	Faint yellow	*Mucor*
4	2	Blue purple	Stolon	+	Sporangiospore	Sphericity	2-7	Black	*Absidia*
5	2	Brown green	Flocculence	+	Sporangiospore	Sphericity	6-13	Black	*Circinella*
6	3	Brown	Panniform	+	Sporangiospore	Oval	8-16	Faint yellow	*Thamnidium*
7	2	Gray	Panniform	+	Sporangiospore	Oval	11-13	Dark brown	*Chaetomium*
8	5	Blue	Panniform	+	Conidium	Rod	1-3	Transparent	*Aspergillus*
9	6	Dark green	Panniform	+	Conidium	Chain	2-7	green	*Penicillium*
10	6	Green	Panniform	+	Conidium	Sphericity	2-5	green	*Trichodema*
11	3	Discrepant	Panniform	+	Conidium	Oval	2-9	Gray	*Cephalosporium*

+, positive; -, negative.

bacteria diversity of *Cassia occidentalis* rhizosphere differed at the different stages of the plant. The physiological activity of plant is generally considered to be closely related to the stage of the plant. Therefore, we considered that the reason for the different biomass of rhizobacteria in plot GT2 might have been due to the special physiological activity of the dovetree growing in GT2, although further researches should be carried out to investigate the physiological activity of the dovetree at different stages or using different tissue.

Strain identification is a time-consuming work although several molecular methods have been established. In the present study, both conven-

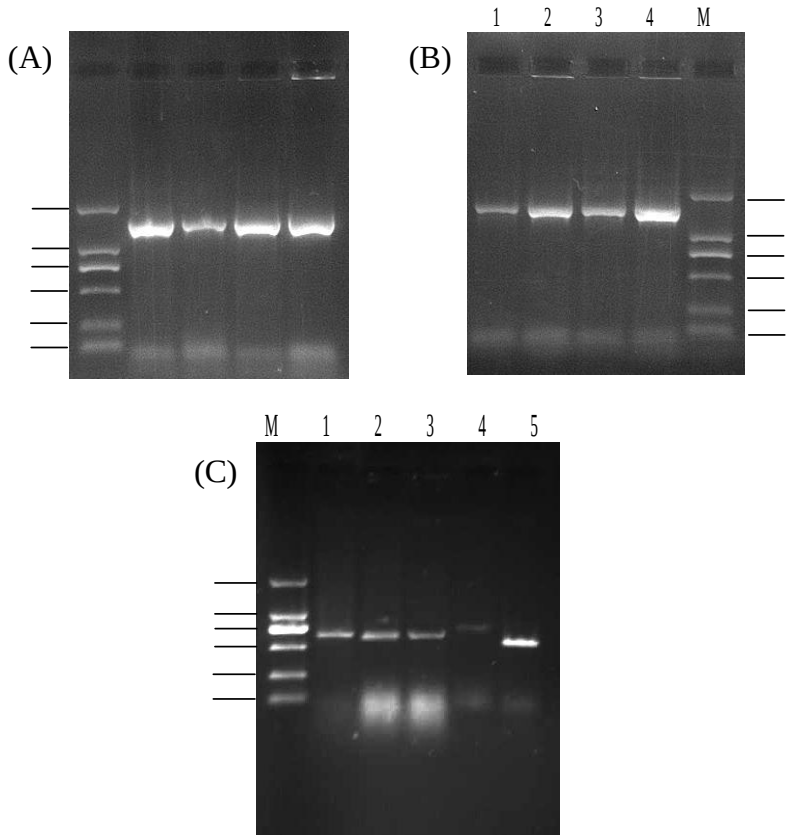


Figure 1. Electrophoretic analysis of colony PCR products. **(A)** lane M, D2000 DNA marker (TINAGEN); Lane 1-4, partial 16s rDNA amplicons of rhizobacteria isolates; **(B)** lanes M: D2000 DNA marker; lane 1-4, partial 16s rDNA amplicons of streptomyces isolates; **(C)** lane M, D2000 DNA marker; lane 1-5, partial 16s rDNA amplicons of fungi isolates. The electrophoresis picture of other colony PCR products was not showed in the paper.

tional methods and molecular methods were used to identify the isolates at the genus level for the assessment of diversity as previously reported (Marie et al., 2010). The results reveal that genus identification can be done using morphology examination and the phylogenetic identification method.

The importance of rhizosphere microbial diversity for maintenance of root health, nutrient uptake, and tolerance of environmental stress has been well recognized (Bowen and Rovira, 1999; Cook, 2002). In this study, a genera of *Bacillus* (36.54%, n=19) was detected as the dominant rhizobacteria. A number of investigators have reported that the genus *Bacillus* has good potential in promoting plant growth (Khurram et al., 2012; Sgroy et al., 2009), which is probably because the *Bacillus* provides a double benefit to the plant: (1) It gradually releases phosphorus (P) from insoluble P complexes though P-solubilization activity, and (2) it improves root growth and root surface area for better uptake of P and other nutrients through ACC deaminase activity. Based on the benefit mechanisms of *Bacillus*, we have concluded that the huge mass of *Bacillus* in the dovetree rhizophere soil should be beneficial to the growth of the dovetree. In this study, 21 cultivable genera were isolated, including nine bacteria genera, 11 fungi genera and one streptomyces genus. Those rhizophere microorganisms constitute a complex rhizophere ecosystem that could significant influence the soil fertility and structure (Lynch and Bragg, 1985). Moreover, it is estimated that 99% of microorganisms observed in nature are typically not cultivated using standard techniques (Amann et al., 1995). Consequently, the rhizophere symbiosis is highly complex and its plant growth promotion mechanism is not fully understood.

In summary, we have concluded that (1) the *Bacillus*, *Lactobacillus*, *Azotobacter*, and *Streptomyces* are the dominant microorganism's genera in the rhizophere soil of the dovetree and that *Bacillus* is the dominant rhizobacteria that might have potential in host growth promotion; (2) the physiological activity of the dovetree could significantly influence the microorganism biomass of the rhizophere soil; and (3) the dovetree has a highly complex rhizosphere ecosystem and further study should be carried out in this area.

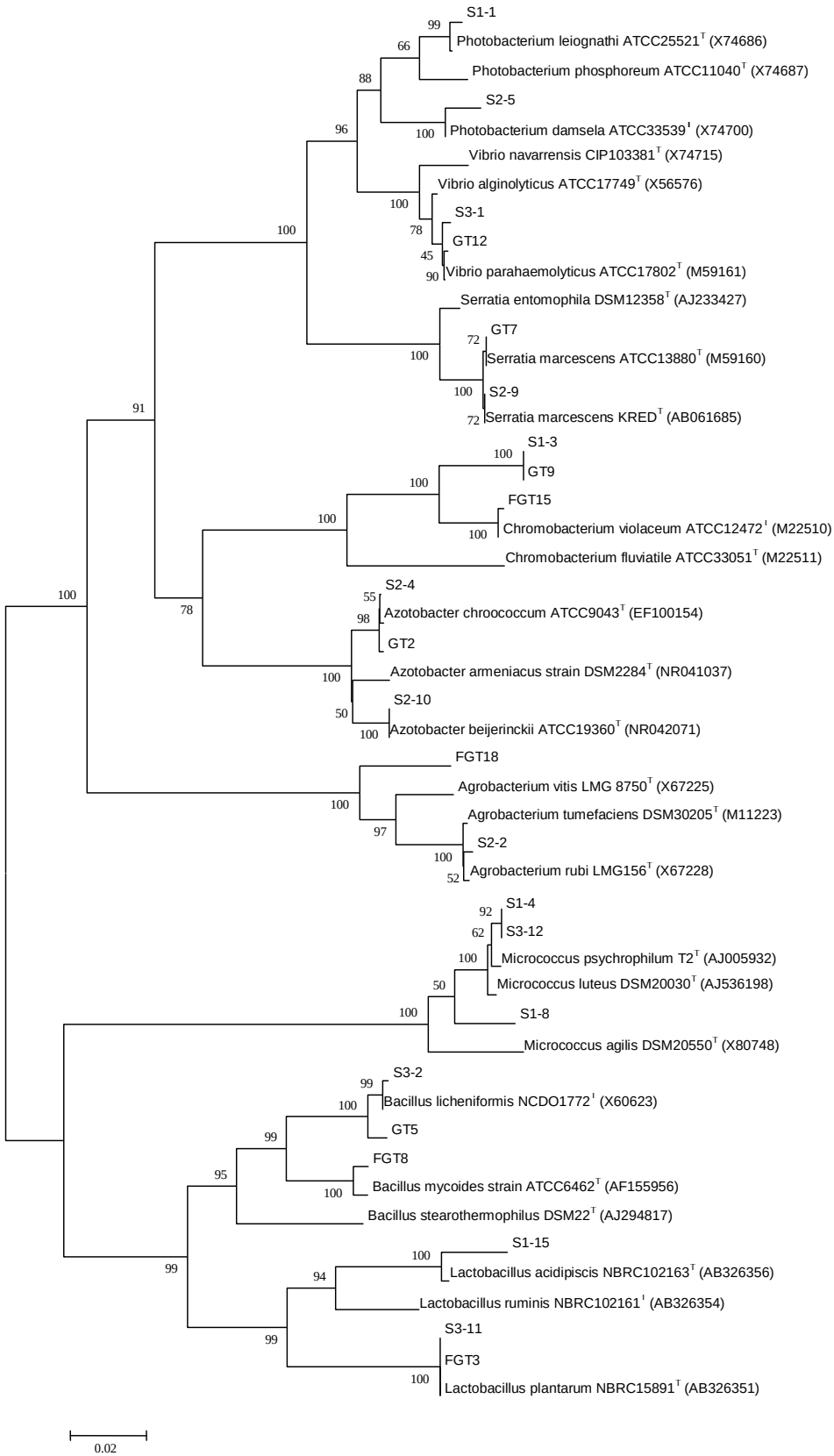
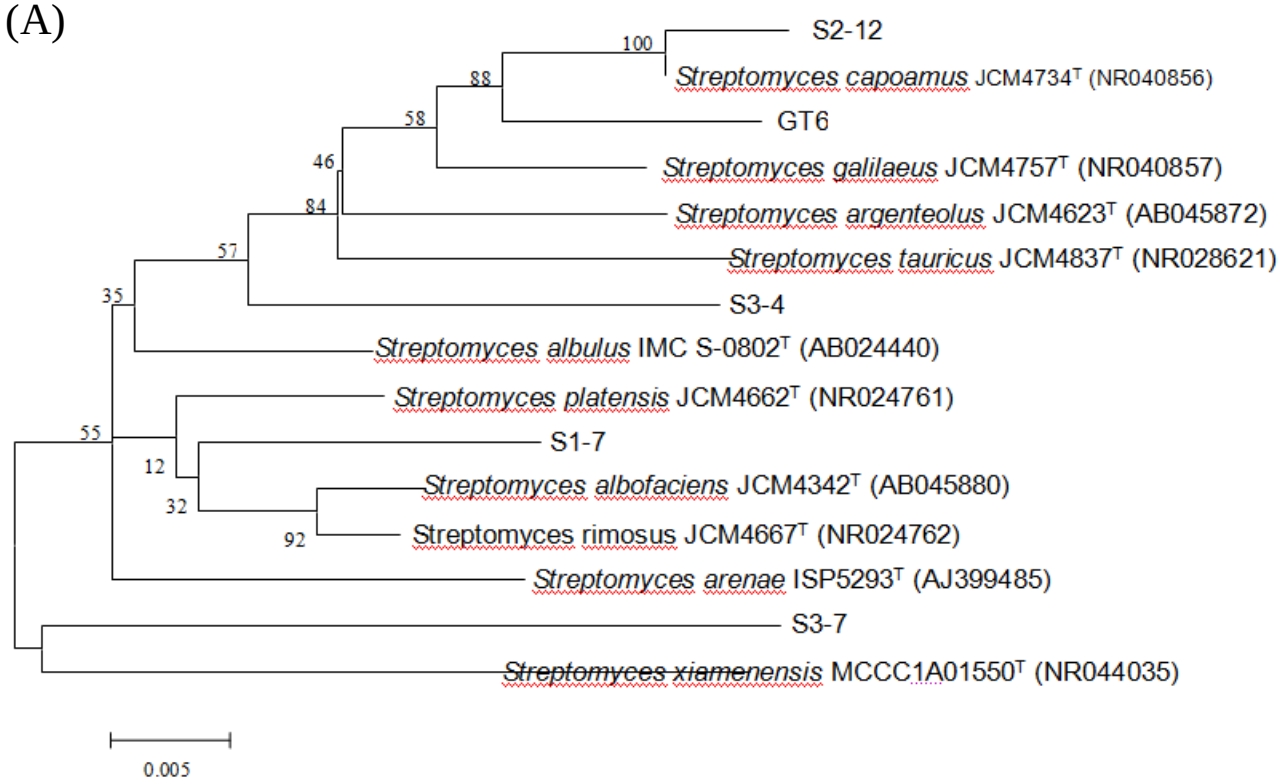


Figure 2. Phylogenetic tree of rhizobacteria isolates. The phylogenetic tree was constructed by Kimura 2-parameter model and Neighbor-Joining method. The robustness of individual branches was estimated by using bootstrapping with 1000 replications and the phylogenetic tree was conformed by the maximum-parsimony method and maximum-likelihood method.

(A)



(B)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. GT6															
2. S2-12	0.022														
3. S1-7	0.045	0.044													
4. S3-4	0.043	0.043	0.047												
5. S3-7	0.058	0.066	0.048	0.070											
6. AB045880	0.044	0.043	0.023	0.034	0.047										
7. AB024440	0.040	0.040	0.027	0.029	0.050	0.017									
8. AJ399485	0.043	0.034	0.041	0.047	0.057	0.037	0.035								
9. AB045872	0.030	0.037	0.039	0.035	0.057	0.037	0.033	0.044							
10. NR040856	0.018	0.005	0.040	0.039	0.061	0.038	0.035	0.029	0.031						
11. NR040857	0.022	0.024	0.042	0.033	0.061	0.040	0.028	0.031	0.020	0.018					
12. NR024761	0.040	0.036	0.027	0.038	0.044	0.019	0.021	0.030	0.036	0.030	0.038				
13. NR024762	0.039	0.041	0.024	0.037	0.042	0.008	0.020	0.035	0.037	0.036	0.042	0.015			
14. NR028621	0.036	0.037	0.040	0.040	0.064	0.040	0.034	0.040	0.029	0.032	0.027	0.039	0.038		
15. NR044035	0.058	0.066	0.054	0.053	0.061	0.044	0.048	0.049	0.060	0.061	0.059	0.048	0.044	0.065	

Figure 3. Phylogenetic tree of streptomyces isolates (A) and Pairwise distance of Streptomyces (B).

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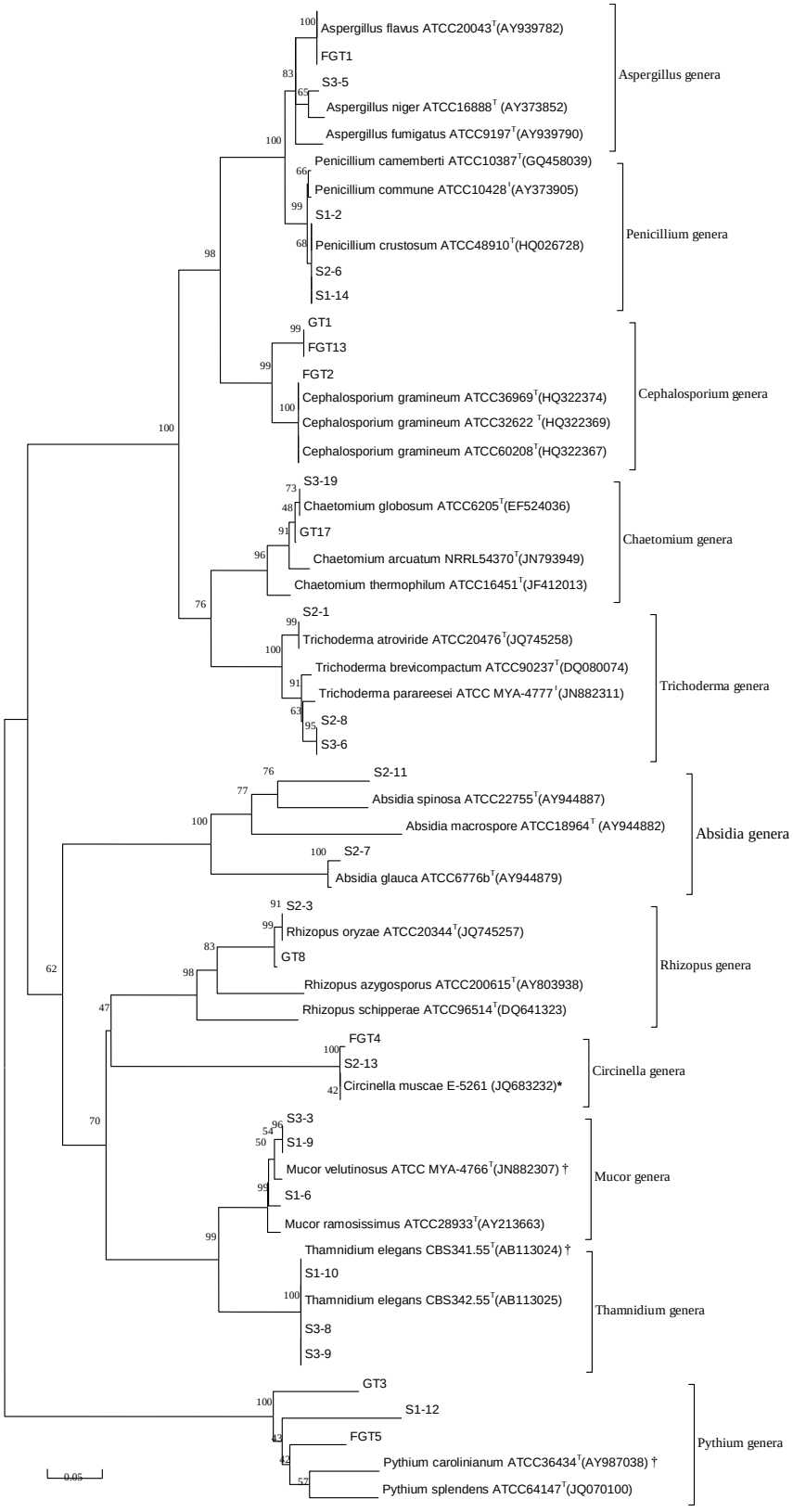

Figure 4. Phylogenetic tree of fungi isolates. The phylogenetic tree was constructed by Kimura 2-parameter model and Neighbor-Joining method. The robustness of individual branches was estimated by using bootstrapping with 1000 replications and the phylogenetic tree was confirmed by the maximum-parsimony method and maximum-likelihood method. *, no typical circinella genera strain is searched in Genbank database, circinella muscae E-5261 isolate was selected to construct the pylogentic tree; †, there were only two typical strains were searched in Genbank database.

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Full Length Research Paper

Water molds *Saprolegnia diclina* (FLO) isolated from eggs of *Carassius carassius* L. in Białystok Rivers, Poland

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Investigations of occurrence of *Saprolegnia diclina* from four limnocrenic springs of rivers within the Białystok where eggs of *Carassius carassius* were the bait were done. Identification of isolates was accomplished on the basis of their vegetative, asexual reproduction, generative organs and by studying the sequencing of the internal transcribed spacer of nuclear ribosomal DNA (ITS1+5.8S+ITS2). *S. diclina* occurred in 29 (60.4%) [10(20.1%) in spring, 4 (8.3%) in summer, 11 (23.0%) in autumn and 4 (8.3%) in winter, 2005] of the 48 examined water samples. The results indicate the sequence comparisons of two ITS nuclear DNA for species identification: *S. diclina.* The results indicate that the sequence of our isolate correspond to *S. diclina*. It is very important that this study represents the first isolation (on the basis of molecular features) of *S. diclina* in fresh waters in Poland.

Key words: *Saprolegnia diclina,* detection, nuclear DNA, eggs, *Carassius carassius*, springs, Poland.

INTRODUCTION

Water moulds constitute a common group of organisms found in a variety of water ecosystems. Some of them are animal or human parasites. In favorable conditions, water mould acts as saprobionts and can assume pathogenic properties, being a potential source of infection (Dick, 2001; Czeczuga et al., 2004a, b; Kiziewicz and Kurzątkowska, 2004; Kirk et al., 2008). In springs of rivers, a lot of representatives of water moulds of the class Oomycetes from Saprolegniaceae family are present (Kiziewicz, 2012).

Identification of family Saprolegniaceae traditionally has relied on the observation of morphological features. Genera of the Saprolegniaceae have been differentiated by their method of zoospore release (Daugherty et al., 1998). Species identification has been more challenging because it requires the presence of the sexual structures, the oogonia and the antheridia. More recently, molecular identification has been accomplished with selected Saprolegniaceae by usage of the internal transcribed spacer (ITS) and 5.8S regions of ribosomal DNA (rDNA) (Leclerc et al., 2000). The most complete molecular phylogeny of this family to date identified 10 genera and 40 species through analyses of ITS and the large ribosomal subunit (LSU) (Leclerc et al., 2000; Petrisko et al., 2008).

There have been relatively few published field investigations of water moulds from family Saprolegniaceae diversity and ecology (Johnson et al., 2002).

The main task of the present study was to make the first complete characterization of a strain of *S. diclina* by using morphological and molecular features and assessing *S. diclina* growth on the crucian carp *Carassius carassius* as bait from four limnocrenic springs of rivers

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Table 1. Distribution and seasonal occurrence of aquatic fungus *Saprolegnia diclina* in 48 samples from four different sites of springs in Białystok (n =3).

Water reservoir		Seasonal occurrence/Number of water samples where fungus was found				
(Name of water reservoir –spring)	Number of collected water samples	Spring	Summer	Autumn	Winter	Together
Dojlidy Górne	12	2	1	3	1	7
Jaroszówka	12	3	1	2	1	7
Cypisek	12	3	1	3	1	8
Pietrasze	12	2	1	3	1	7
Total number of samples	48	10	4	11	4	29
Percentage	100	20.1	8.3	23.0	8.3	60.4

situated in Białystok.

MATERIALS AND METHODS

Study area

We conducted investigations about the occurrence of fungus *S. diclina* in water in several springs situated in Dojlidy Górne, Jaroszówka, Cypisek and Pietrasze at Białystok town during the spring, summer, autumn and winter of 2005.

Spring Dojlidy Górne (53°06'N, 23°12'E) and Spring Pietrasze (53°10'N, 23°9'E) in Biała River are located at the eastern and north part of Białystok. Spring Jaroszówka (53°10'N, 23°11'E) and spring Cypisek (53°10'N, 23°11'E) belong to Jaroszówka River and are located in the north part of Białystok.

These springs are characterized as Limnokrenic types, with an artificial basin: area from 0.380 to 0.290 km^2, width 0.65 m, depth 0.12 m, discharge from 2.4 to 2.5 dm^3 s^{-1}, and surroundings characterized by cultivated fields, herbaceous vegetation, trees and buildings.

Microbial analyses

Isolation of the fungus

Microbial analyses were made in the laboratory of the Department of General Biology, Medical University of Białystok, Poland and in the laboratory of the Real Jardin Botanico de Madrid, Spain from 2005-2006. For the microbial analysis of fungi, samples of water were collected from each site described above.

Samples were processed in the laboratory by routine methods commonly used to isolate these organisms. Bait method by using eggs of the crucian carp *C. carassius* and hemp seeds *Cannabis sativa* L. was applied to isolate *S. diclina* from the springs. Water samples (100 ml) from each site were homogenized and four aliquots of 25 ml were placed in Petri dishes of 9 cm diameter with sterile baits. Dishes were stored in the laboratory at room temperature (20-23°C) for 4-5 days (Seymour and Fuller, 1987). The colonized fragments of crucian carp eggs and hemp seeds, were transferred to new Petri dishes which contained sterilized, filtered spring or distilled water and crystalline penicillin C (100 mg L^{-1}) to inhibit bacterial growth. Dishes were microscopically examined weekly for up to three weeks in order to identify water moulds at the level of genus or species. The isolate was placed onto agar medium - PG1. To prevent bacterial growth, penicillin C was added to the agar to a final concentration of 100 mg L^{-1}. A piecem of infected bait was placed on the top of the agar and into a previously placed glass ring 3 cm diameter to protect the growing fungus-like organisms (FLO) from bacteria. The isolates were maintained on agar medium - PG1 and stored in the culture collection of the Real Jardín Botánico de Madrit, Spain. Morphological characters of asexual and sexual structures and measurements were made microscopically on material mounted in water. FLO were successively observed under an optic microscope Olympus BX 51 (100 and 400x magnification). All isolates were characterized and identified according to Unestam (1965), Batko (1975), Seymour and Fuller (1987) and Alexopoulos et al. (1996).

DNA extraction and PCR amplification

For DNA extraction, mycelium was grown as a drop cultures (Cerenius and Söderhall, 1985) and from them, genomic DNA was extracted using an E.Z.N.A.–Fungal DNA Miniprep Kit (Omega Biotek, Doraville, USA) as described by Martín and García – Figueres (1999). DNA fragments containing internal transcribed spacers ITS1 and ITS2 including 5.8S gene of the nuclear DNA was amplified with primer pairs ITS5/ITS4 (White et al., 1990) primers as described by Martin et al. (2004). Nucleotide BLASTN searches with option Standard nucleotide BLAST and BLASTN 2.6 were used to compare the sequence obtained against the sequences from the National Centre of Biotechnology Information (NCBI) nucleotide databases.

The new consensus sequence has been deposited in the EMBL data –base under accession number 1289 (*Saprolegnia diclina*).

RESULTS AND DISCUSSION

In the present study, *S. diclina* was isolated from water belonging to several springs in the area of Białystok, Poland and using crucian carp eggs and hemp seeds as bait. The study showed the occurrence of fungus-like organisms (FLO) *S. diclina* in samples of different water reservoirs. Identification of isolates was accomplished on the basis of their vegetative, asexual reproduction, generative organs and by studying the sequencing of the internal transcribed spacer of nuclear ribosomal DNA (ITS1+5.8S+ITS2).

As shown in Table 1, *S. diclina* occurred in all the four springs of examined in 29 (60.4%) of the 48 examined water samples [10 (20.1%) in spring, 4 (8.3%) in summer, 11 (23.0%) in autumn and 4 (8.3%) in winter, 2005]. The

isolate was characterized by studying the sequencing of the internal transcribed spacer of nuclear ribosomal DNA (ITS1+5.8S+ITS2). The results indicate the sequence comparisons of two ITS nuclear DNA for species identification: *S. diclina* 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence. Results indicate that our isolate corresponded to the species *S. diclina* Humhrey ATCC 90215 (AY455775, version AY455775.1; GI: 38156477) Oomycetes or Oomycota, which are heterokont fungus-like organisms (FLO) (phylogenetically not true fungi) in the Kingdom Chromista/Stramenopila (Alexopoulos et al., 1996; Dick, 2001; Kirk et al., 2008).

Species of the genus *Saprolegnia* are generally considered saprobes that live on decayed plant or animal debris. *Saprolegnia* is often found in polluted and marshy waters. However, this genus also includes parasites of freshwater animals and their eggs, and some of these are responsible for economically important diseases by affecting farmed and wildlife populations of aquatic animals (Söderhäll et al., 1991; Kiesecker et al., 2001; van West, 2006; Fernández-Benèitez et al., 2008). Some of species from Saprolegniaceae such as *Saprolegnia* sp. and several different genera are parasites of arthropods, crustaceans, mosquito larvae, eggs of freshwater fish and eggs, and tadpoles of amphibians at their embryonic or larval stages and also they were described as parasites of reptiles (Seymour, 1984; Westman, 1991; Vennerstrom et al., 1998; Kitancharoen et al., 1995; Hulvey et al., 2007; Fernández-Benéitez et al., 2008; Eli et al., 2011).

Mycotic infections associated with family Saprolegniaceae are widely reported in freshwater fish; however are rarely found in brackish water (Czeczuga and Woronowicz, 1993; Hussein and Hatai, 2002).The disease saprolegniasis is caused by freshwater fungi usually from the genus *Saprolegnia* which is generally considered as opportunistic pathogens for fish and their eggs (Bruno and Wood, 1999; Bangyeekhun et al., 2003; Kiziewicz et al., 2011). Saprolegniasis is a continuing problem for aquatic animal populations. *Saprolegnia ferax, S. diclina* and *S. parasitica* are known to be pathogens to cold water fishes. Any species of fish that are intensively cultured and captured are at risk of suffering fungal diseases (Czeczuga, 1994; Blazer et al., 2002). Among them, *Saprolegnia ferax* and *S. parasitica* are the most dangerous, causing the death of whole populations of many fish species in certain water basing as is the case of breeding populations of Pacific salmon (Czeczuga and Muszyńska, 1996) and the Atlantic salmon *Salmo salar* in Great Britain and Poland (Stuart and Fuller, 1968; Czeczuga et al., 2011). Some studies revealed mortality around 70-90% of incubated spawns of acipenserids (Dudka et al., 1989). The most common species involved in disease outbreaks of saprolegniales is *S. diclina*. It can occur in salmonids (Kitancharoen and Hatai, 1998; Fregeneda-Grandes et al., 2007). In the present study, we were successful in isolating *S. diclina* from water of springs - located in Białystok, Poland using eggs of crucian carp as bait. Chukanhom and Hatai (2004) identified fungal infections of common carp (*Cyprinus carpio*) eggs in Thailand. The fungus-like organisms from this genus are regarded primarily as a saprothroph - an organism that lives on dead organic matter, but during infection; it proved to be the most efficient at infecting fish eggs. They grow and penetrate into the cell wall reducing water flow and enzyme secretion which lead to death of eggs (Fadaeifard et al., 2011). Other species of *Saprolegnia* have been observed growing on the spawn of amphibian and reptilian species as well (Czeczuga et al., 1998; Fernández-Benéitez et al., 2008; Petrisko et al., 2008). In contrast to some general assumptions of the opportunistic nature of the pathogenicity of *S. diclina*, recent studies appear to indicate that *S. diclina* is adapted to colonize as a saprothroph egg of salmonids. *S. diclina* is also frequently observed and isolated from different species of adult fish and their eggs (Czeczuga and Muszyńska, 2000; Fregeneda-Grandes et al., 2007). The fungus also observed growing on eggs of the sea trout *Salmo trutta* m. trutta in River Biała, Krasna and Supraśl near Białystok in Poland. The investigated eggs were collected from 60 females of Atlantic salmon caught during their spawning migration in Darłowo town on the River Wieprza (wild form), and Świbno town on the River Vistula (wild form), and in fresh water in hatcheries at Miastko town (farmed form) (Czeczuga et al., 2005). The ubiquitous presence of one or more species of *Saprolegnia* indicates that these fungi might have a role in the biological recycling at farm fisheries. According to Willoughby (1994) FLO which belongs to genus *Saprolegnia* can be one of the fungal causes in freshwater fishes and their eggs. Our results showing the occurrence of *S. diclina* on eggs of *C. carassius* confirm this. All morphological, physiological and genetic features studied were identical, and then the isolates were considered to be from those of the same strain. This study represents the first isolation of *S. diclina* in fresh waters in Poland and also this study is the first one that describes *S. diclina* by using molecular features (ITS rDNA) in Poland.

Conclusion

In the present study, we successfully isolated fungus-like organisms (FLO) of *S. diclina* from spring water of Białystok and determined it by using eggs of crucian carp as bait. The results show that the sequence of our isolate correspond to the species *S. diclina* using the sequence comparisons of two ITS nuclear DNA for species identification.

Thus, this study represents the first isolation (based on molecular analysis) of *S. diclina* in Poland fresh waters.

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Full Length Research Paper

The antibacterial activity and some trace elemental compositions of the extracts of *Piliostigma thonningii*, a widely used ethnomedicinal tree in Minna, Nigeria

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The study on antibacterial activity of the extracts of the leaf, stem bark and roots of *Piliostigma thonningii* (Schum) Milne-Redh (Ceasapinaceae) (an underexploited plant in Nigeria) at 25 mg/cm^3 each using agar diffusion method, was carried out. The samples were collected from Gidan Kwano Campus of Federal University of Technology, Minna (North Central Nigeria). The extracts of these parts exhibited appreciable activities against the test organisms: *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Streptococcus pyogene, Proteus vulgaris, Salmonella typhi* and *Escherichia coli.* The results obtained in this study corroborated the local medicinal applications of the plant by traditional practitioners in this part of the country.

Key words: *Piliostigma thonningii*, antibacterial activity, elemental composition, *Escherichia coli*.

INTRODUCTION

Generally, persistent contamination of media by pathogenic microorganisms leads to various disease conditions in human life and in recent years, the antimicrobial efficacy of many plant extracts and metals has been investigated against microorganisms including *Staphylococcus aureus*, *Klebsiella* spp., *Escherichial coli*, *Klebsiella* spp., *Streptococcus pyogene*, *Bacillus subtilis* and *Pseudomonas aeruginosa* (Harris et al., 2002; Kone et al., 2004; Drevensek et al., 2006; Jigna and Sumitra, 2007).

B. subtilis, a ubiquitous rod shaped peritrichous Gram-positive bacterium commonly found in water, soil, air and decomposing plant residues (Ashlea et al., 2008), is used in fermentation processes although it has been reported to produce an extracellular toxin (subtilism) which causes allergic reactions in individuals repeatedly exposed to it (EPA, 1997). *Proteus vulgaris* on the other hand, is a peritrichous Gram-negative bacillus of the family Enterobacteriaceae which inhabits the intestinal tracts of humans and animals. It is found in soil, water and faecal matters especially those of patients with compromised immune systems. It is a multidrug resistant bacterium that causes urinary and wound infections (Yu et al., 2011). *P. aeruginosa* is a Gram-negative aerobic rod shaped bacterium found in soil, water, skin, flora and most man-made environments (Stojek et al., 2008; Philip et al., 2009). It causes diseases like endocarditis, bacteremia, central nervous system, eye, ear, bone and joint infections. *Salmonella typhi* on the other hand, is a Gram-negative microbe that causes typhoid fever, a disease

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that affects over 16 million people in the world annually (Shangkuan and Lin, 1998; Parkhill et al., 2001; Ganai et al., 2009). It is a multiple drugs resistant bacterium (Cristina and Claudia, 1994). *S. aureus* is a Gram-positive non-spore forming facultative anaerobic bacterium that has high multidrug resistance (Harris et al., 2002; Waters et al., 2011). It has been responsible for causing skin and soft tissue, blood and cardiovascular system diseases. Also, *E. coli* commonly found in the lower intestine of warm blooded organisms is also a gram-negative facultative anaerobic bacterium. It causes uncomplicated infections of the urinary tract (Gabriela et al., 2011) while *Klebsiella pneumonia* is a Gram-negative, non-motile, encapsulated rod-shaped bacterium of the family Enterobacteriaceae which induces septicemia, pneumonia, pneumococcal meningitis and peritonitis (Podschun and Ullmann, 1998) whereas *Streptococcus pyogene* is responsible for many childhood diseases (Fox and Rohovsky, 1975; CWB Info.com, 1999; Clark, 2000; CDC, 2006; Microbiology Bytes, 2007).

Metals play a wide variety of roles in biological systems acting as cofactors in enzymes and as components of drugs for the treatment of various diseases and conditions (Piedad et al., 2008). It is in this wise that iron plays a key role in the lives of living organisms and a metal that is required for their growth in addition to the critical role it plays in the pathogenesis of infections (Yukihiro et al., 2007). Iron is therefore, used either singly or in combination to combat pathogenic microbial growth in media (Saeed and Farshid, 2008; Scott et al., 2009; Joshi et al., 2011). Studies on the use of various forms of copper as an antimicrobial agent either as a single entity or in combination with other metals or chemical substances have been reported (Yu-Sen et al., 1996; Copper Development Association, 2004; Aisha, 2005; Jayesh et al., 2008; Wheeldon et al., 2008). In these forms, it plays significant role in improving public health functioning at low concentrations as a bacteriostat, fungicide, antivirus, antimould, antialgae in drinking water and wood preservative (Mike and Craig, 2008; Rabin et al., 2010). It is also one of the relatively small groups of metallic elements essential to human health which is required in trace amounts for the growth and functioning of organisms (Rabin et al., 2010). In addition, magnesium is required for the growth of organisms apart from the fact that it plays an important role in the pathogenesis of infections. Thus, magnesium and most especially its compounds or complexes are employed in chemotherapy for the control of microorganisms (Drevensek et al., 2006; Lellouche et al., 2011). Chromium is a heavy metal that plays a key role in the microbial activity of plant extracts by making these phytochemicals unavailable for antimicrobial activities (Ata-Ullah et al., 2011). At certain concentrations however, this metal could also act as an antimicrobial agent. Zinc plays a key role in the lives of living organisms and is a metal that is required for their growth. It occurs in small amounts in almost all igneous rocks with its principal ores being sulphides like sphalerite and wurtzite. However, taking too much of this metal into the body affects human health. This is because it binds to particulate matter and its soluble species are readily available for biological reactions thus making the metal a very toxic one. Therefore, this metal also plays a vital role either alone or in its combined forms in the pathogenesis of infections.

Numerous studies have shown that medicinal plants are the oldest health care products with proven efficacy serving as the basic components of several hard drugs, analgesics, anaesthetics, antibiotics, anti-cancer, anti-parasitic, anti-inflammatory, oral contraceptives and diuretic drugs (Sofowora, 1982; WHO, 1995). It has also been shown that 80% of world's populations rely entirely on local medicine made almost exclusively from plants especially in developing countries like Nigeria where multiple drugs resistance to several orthodox antibiotics is being experienced (WHO, 1995; Akinpelu et al., 2000; Saidu et al., 2000; Adiaratou et al., 2005). For instance, *Abrus precatorius* L. which belongs to the family Fabaceae, has been reported to be used for the treatment of cough, convulsion, rheumantism and as a labour inducer while the fruits and leaves of *Aframomum melegueta* K. Schum of the family Zingiberaceae are used for the treatment of catarrh, small and chicken poxes (Ige, 2011). Furthermore, plants such as *Amaranthus spinosus* and *Annona senegalensis* Pers are used for the treatment of piles, abdominal pains and snake bites (Ige, 2011). Also, the use of *Allium sativum* to reduce the cholesterol levels and boost the immune system of the human body has been reported (Oyeleke et al., 2008), while ginger and garlic have been reported to have inhibitory effects on the growth of coliform bacteria as well as *E. coli*, *Proteus vulgaris*, *S. aureus* and *Salmonella* spp. (Kennedy et al., 2007).

Piliostigma thonningii (Schum) Milne-Redh (Ceasalpinaceae) is found growing abundantly as a wild uncultivated small tree in many parts of Nigeria such as Minna, Zaria, Bauchi, Ilorin, Plateau, Lagos and Abeokuta (Keay, 1989). It is a leguminous plant belonging to the family Caesalpinaceae, a family that comprises trees and shrubs. Our attention was drawn to this plant because of several claims on its enormous applications as an ethnomedicinal plant used to cure several diseases including the common malaria, dysentry, fever, infectious respiratory ailment, snake bite, hookworm, hepatobiliary ailments, hydropsy, sterility, rachitis and skin diseases (Tira-Picos et al., 2010).

Although, several countries have already recognized the importance of traditional medical applications in their health care delivery systems (WHO, 1995), traditional healers in Nigeria have not yet been given the position they deserve despite the fact that a large proportion of the population still rely heavily on traditional practitioners, including traditional birth attendants (where traditional birth attendants assist up to 95% of all rural birth), herbalists, bonesetters and a host of local medicinal plants to

satisfy their primary health care needs. These different parts of this plant in order to establish or otherwise debunk the medicinal values of *P. thonningii*.

MATERIALS AND METHODS

Fresh samples of the leaves, roots and stem barks of the plant were randomly taken from the premises of Gidan Kwano Campus of the Federal University of Technology, Minna, Niger State, Nigeria. This plant in the area is found growing freely as a wild small tree. Samples of the root, stem bark and leaves of the plant were collected in three batches between the months of May and July, 2011 and the plant was identified by Professor Z. I. E. Ezenwa of the School of Agriculture and Agricultural Technology, Department of Soil Science, Federal University of Technology, Minna, Nigeria. After sampling, the samples were taken to the laboratory, detached from the twigs, carefully sponged with water containing little detergent and well rinsed with deionized water to remove the surface contaminants. They were then dried at ambient temperature in the laboratory to avoid heat destruction of the active components of the samples. The dried samples were ground into study explored the antibacterial activities of extracts from fine powder by pounding mechanically with clean sterile pestle and mortar to increase the surface area. The ground samples were kept in airtight polyethene bags for further use. When required, the dried ground samples were extracted with four different solvents namely water, petroleum ether, chloroform and methanol. Each sample was separately extracted by using the solvents in turn. 150 g of the powdered sample (in one extraction) was put in a thimble which and introduced into a soxhlet extractor and refluxed for several hours. At the end of all extractions, 9 petroleum ether, 6 methanolic, 3 each of the cold maceration and boiled aqueous extracts of the roots, stem bark and leaves were obtained, respectively. The solvent of each extract was evaporated on the water bath and the residues, after cooling, were covered with aluminium foils for subsequent analysis.

Phytochemical analysis

The crude extracts of the samples were analyzed for their phytochemical constituents using standard methods described by Evans and Trease (1989), Sofowora (1982) and Sofowora (1986).

Antimicrobial analysis

Microorganisms

Antibacterial activity of the obtained plant extracts was evaluated against *S. pyogene, P. aeruginosa*, *S. typhi*, *S. aureus*, *E. coli, Klebsiella* spp., *P. vulgaris* and *B. subtilis* using agar diffusion method. The strains were obtained from the Department of Microbiology, School of Science and Science Education, Federal University of Technology, Minna, Nigeria and were cultured on a nutrient agar. After activation from stock culture, microorganisms were maintained in the nutrient broth until required for use. Cultures were prepared by growing the strains at 37°C for 24 h in the agar broth and repeatedly sub-cultured in order to obtain pure isolates. These were then Gram stained for proper identification and inoculated into agar slants and stored at 40°C for further use. Before use, each of the test organisms was seeded into 25 cm^3 of the nutrient agar to produce a culture whose turbidity was 0.5 on the McFarland scale corresponding to a concentration of 1.0×10^8 colony forming units per cm^3 (Saeed and Farshid, 2008). For pre-diffusion, the plates were kept at room temperature for 2 h then incubated at 37°C for 24 h. A total of 60 plates which were randomly divided into ten test groups with six plates each with each of the test organism tested were used. Under identical incubation and aseptic conditions, positive and negative controls with and without inoculums were prepared.

Agar diffusion test

In this study, nutrient agar which is one of the most widely used *in vitro* media for the evaluation of antibacterial activity that identifies the extracts more likely to have antimicrobial effects on common microorganisms which are highly influenced by the diffusion ability of the extract across the medium was employed. 28.0 g of the powdered commercially prepared nutrient agar was weighed and dissolved in 1 dm^3 of sterile distilled water and this was sterilized at 121°C for 15 min. 25 cm^3 of this was then dispensed into sterilized single-layered 2 × 10 cm Petri-dishes. Uniform cavities each of 4 mm diameter were drilled at equal intervals in the poured agar by means of a sterile copper loop after 24 h. This was then inoculated with the test bacteria using swab sticks at 37°C for 3 h. The cavities were then filled with the plant extracts immediately after reconstitution (one extract in one hole). This was allowed to stay for 1 h to allow the extracts to be absorbed by the agar. The absorbed nutrient agar was kept in an incubator at 37°C for incubation for 24 h.

Data recording

The diameter of bacterial growth inhibition zones was measured with a millimeter ruler whose accuracy was 0.5 mm. These readings were taken in triplicates in order to minimize error.

Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) for the mean concentrations of the minerals as well as the zones of growth of inhibition among the test organisms using Duncan's multiple range tests and were reported as mean ± SE (standard error) of triplicate values. SPSS 16.0 statistical package was used and the statistical significance of the data obtained was established at $p < 0.05$ or 95% confidence while differences among values were established using post hoc tests.

RESULTS

The antimicrobial activities of the test materials as indicated in Table 1 were determined by the means ± SE (standard errors) of the zones of inhibition in millimeters on all the microorganisms after 24 h. All bacterial strains were inhibited by the test materials with varying degrees of susceptibility. These results revealed that the antimicrobial effect of the aqueous extracts (AE) covered the widest range of microorganisms upon which they were active although the highest zone of inhibition (35 mm) on *E. coli* was exhibited by the petroleum ether extract (PEE) of the roots of this plant. The order of inhibitions of the growth of the test organisms for the root extracts, in decreasing order, was; petroleum ether extract (PEE), methanolic extract (ME), aqueous extract (AE) and chloroform extract (CE) while for the stem extracts, the order was PEE, ME, AE and chloroform extract (CE). The zones of inhibition of growth of organisms by the leaves extracts was in the order PEE = ME > AE > CE. None of

Table 1. The antimicrobial activities (zones of inhibition in mm) of the extracts (100 mg/ml) of the various parts of *P. Thonningii*.

Bacteria	Zones of inhibition (mm)											
	Water			Pet . ether			Methanol			Chloroform		
	Roots	Stem bark	Leaves	Roots	Stem bark	Leaves	Roots	Stem bark	Leaves	Roots	Stem bark	Leaves
Escherichia coli	8.00±0.58^{a}	-	-	35.00±0.72^{b}	-	-	-	-	-	-	-	-
Klebsiella spp	16.00±0.88^{b}	20.00±0.58^{d}	18.00±0.59^{c}	30.00±0.26^{e}	-	-	-	-	-	10.00±0.54^{a}	-	-
Streptococcus pyogene	12.00±0.57^{a}	16.00±0.20^{b}	23.00±0.15^{c}	15.00±0.58^{b}	-	25.00±0.58^{d}	15.00±0.38^{b}	-	-	-	-	-
Staphylococcus aureus	20.00±0.58^{d}	15.00±0.61^{c}	10.00±0.21^{a}	10.00±0.32^{a}	15.00±0.42^{c}	-	13.00±0.54^{b}	23.00±0.48^{e}	-	-	15.00±0.38^{c}	10.00±0.58^{a}
Proteus vulgaris	15.00±0.58^{b}	16.00±0.23^{b}	10.00±0.12^{a}	-	-	-	-	-	-	-	-	-
Salmonella typhi	6.00±0.58^{a}	-	10.00±0.58^{b}	-	34.00±0.32^{c}	-	-	-	-	-	-	-
Bacillus subtilis	5.00±0.00^{a}	6.00±0.24^{a}	10.00±0.24^{b}	-	-	-	17.00±0.28^{c}	-	-	-	-	-
Pseudomonas aeruginosa	6.00±0.00^{a}	-	-	-	-	-	-	-	26.00±0.32^{c}	-	10.00±0.24^{b}	-

Values are presented as means±SE of three determinations; values with the same superscript in the same row are not significantly different at $p > 0.05$, - = not active, spp = species, pet. = petroleum.

Table 2. The mineral composition of the extracts of various parts of *P. thonningii* (in mg/kg).

Mineral	Root	Stem bark	Leave
Copper	0.23±0.02^{ab}	0.21±0.01^{a}	0.24±0.01^{b}
Chromium	1.71±0.01^{a}	3.50±0.02^{b}	3.50±0.02^{b}
Magnesium	8.02±0.01^{a}	9.13±0.02^{c}	8.12±0.02^{b}
Iron	2.43±0.03^{c}	1.52±0.02^{a}	1.62±0.02^{b}
Zinc	1.21±0.01^{b}	1.10±0.01^{a}	1.64±0.04^{c}

the stem extracts inhibited the growths of *E. coli* but *B. subtilis* was mildly affected by aqueous extract of the stem bark. Also, for the various leaves extracts, only the aqueous extract inhibited the growth of *S. typhi*. The general order of the sensitivities of these bacteria to the various plant parts extracts in this work was *S. aureus* > *S. pyogene* > *Klebsiella* spp. > *B. subtilis* > *S. typhi* > *P. aeruginosa* > *P. vulgaris* > *E. coli*. The copper contents of the three parts of *P. thonningii* significantly differed from one another (at $p < 0.05$) although the chromium contents of the stems and leaves were not significantly different (at $p > 0.05$) (Table 2). The magnesium content of the stem-bark of this plant was the highest followed by that of the leaves while the roots had the lowest value. The iron content of the roots was the highest followed by that of the leaves while that of the stem-bark was the lowest ($p < 0.05$). Furthermore, the zinc content of the leaves was the highest while that of the stem bark was the lowest (at $p < 0.05$).

DISCUSSION

In many countries of the world, especially the developing ones, infectious diseases are the leading cause of death and despite the existence of many antibiotics to fight them, antibiotic resistance as well as substandard antibiotics in the developing countries, the control of which has become a global concern (Jigna and Sumitra, 2007). The emergence of these multidrug-resistant pathogens has, in no small measure, affected the clinical effi-

cacy of many of the existing antibiotics. Hence, many infectious diseases are now being treated with herbal remedies throughout the world either as pure compounds or as standardized plant extracts which provide unlimited opportunities for the treatment of ailments caused by the new drug resistant microorganisms. This has thus led the search for more plants that have new antimicrobial components for the treatment of new and re-emerging infectious diseases. Therefore, this research work focused on the folk medical applications of this plant in order to establish its potential antimicrobial activity. Therefore, the survival of these bacteria in different extracts of the plant was compared and from the results, the plant extracts inhibited the growth of various test bacteria at varying degrees which was an indication that the plant possesses active ingredients that can be used to control the growth of these organisms if appropriate concentrations of the extracts are administered. The zones of inhibition of the growths of the microorganisms employed in this work were comparable with those reported for the various extracts of the leaves of *Cassia ocidentalis* by Sadiq et al. (2012). In their work, it was observed that 120 mg/ml of the ethanolic and water extracts were not active on the growth of *S. aureus*, various extracts of *P. thonningii* were able to inhibit the growth of this organism in this work. It was also reported that the respective zones of inhibition of the growth of *S. typhi* by the ethanolic and aqueous extracts of *C. ocidentalis* leaves were 18 and 17 mm which were higher than the respective 6.0 ± 0.58 and 10.00 ± 0.58 mm obtained in this work for the root and leaves aqueous extracts but lower than the 34.00 ± 0.32 mm obtained for the stem bark PEE. However, the respective 16 and 21 mm zones of inhihibition of the growth of *E. coli* by the aqueous and methanolic extracts of the leaves and stem bark of *Ficus capensis* reported by Oyeleke et al. (2008) were higher than the respective 8.00 ± 0.58 and 35.00 ± 0.72 mm obtained for the aqueous and petroleum ether extracts used in this work.

In this study, all the extracts from the three plant parts, except the PEE and ME of the leaves on one hand and CE of the roots on the other hand, were active against the growth of *S. aureus*. In addition, the aqueous extracts of the various parts of *P. thonningii* showed the widest spectrum of growth inhibition on the test organisms probably because of the synergic antimicrobial activities of the total metallic contents of the aqueous extract which was the phase expected to have the highest content of these metals. The result of this study also showed that the aqueous and petroleum ether extracts of the roots inhibited the growth of *E. coli* showing that this plant can likely serve as a good antimicrobial remedy for combating the diseases caused by this bacterium. This thus justified the use of various parts of this plant in ethno-medical preparations in this part of the world for the treatment of such ailments as diarrhoea, abdominal pains, nausea and as a wash, especially for women with urinary tract infections. Furthermore, it was observed that only the aqueous extracts of the three parts of the plant inhibited the growth of *P. vulgaris*. This indicated that the active components of this plant against this microorganism were likely present in reasonable quantities only in the aqueous extract. Furthermore, since the heavy metals analyzed in this study were present in aqueous phase, they might have had synergic effects on the antimicrobial properties of these extracts especially towards the above mentioned bacterium since some of them have been shown to exhibit antimicrobial activities in various forms (ATSDR, 2011).

The different antimicrobial activities exhibited by the various extracts of *P. thonningii* in this study were in line with the findings of Asuzu et al. (1999), Akinpelu et al. (2000) and Adiaratou et al. (2005) on this plant.

Conclusions

The results of this investigation indicated that *P. thonningii* extracts contained some substantial amounts of phytochemicals that exhibited good antibacteria active-ties and these might have supported the various folk applications of different extracts of this plant to cure ailments from time immemorial. These findings might have also provided research data base for this plant which, hitherto, has been very scanty in this part of the country.

Finally, since some of the extracts employed in this work were able to inhibit the growth of some multiple-drug resistant bacterial strains, *P. thonningii* could be useful in the search for new clinically useful antimicrobial agents.

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Full Length Research Paper

Colonization study of antagonistic Pseudomonas sp. in *Vanilla planifolia* using green fluorescent protein (GFP) as a marker

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Vanilla (*Vanilla planifolia* Andrews) is a high value crop cultivated for the production of Vanillin, one of the most valuable flavoring commodities in the food and beverage industry worldwide. Vanilla cultivation is severely hampered by the prevalence of various fungal diseases. *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotium rolfsii* were isolated from naturally infected vanilla plants and an attempt was made to curtail the spoilage caused by the isolated fungal pathogens using biocontrol agents. *Pseudomonas fluorescens* P7 and *Pseudomonas putida* P4 were isolated from vanilla rhizosphere soil and checked for their capability to control fungal pathogens of vanilla both under *In vitro* and *in vivo* conditions. The endophytic colonisation ability of the selected rhizosphere bacteria were evaluated after genetically tagging them with a constitutively expressing green fluorescent protein gene (gfp). The green fluorescent endophytic bacteria were observed within the plant tissue when cross sections of the petiole were viewed under the confocal laser scanning microscope. The bacterial isolates were effectual in controlling the selected fungal pathogens of vanilla. The gfp- tagged *Pseudomonas* sp. was populated within the intercellular spaces of the vanilla leaves one week after its foliar spraying.

Key words: Biocontrol, confocal laser scanning microscope, endophytic bacteria, *Fusarium oxysporum*, *Rhizoctonia solani, Sclerotium rolfsii.*

INTRODUCTION

Vanilla (*Vanilla planifolia* Andrews, Syn. *Vanilla fragrans* saletest. Ames) is an herbaceous perennial, climbing orchid (Fa. Orchidaceae). Vanilla is the second most expensive spice after Saffron and is cultivated mainly for the production of Vanillin (4 - hydroxy3-methoxy benz-aldehyde) (Walton et al., 2003), one of the most valuable flavoring commodities in the food and beverage industry worldwide (Besse et al., 2004; Divakaran et al., 2006). In India it is grown in an area of 2545 hectares covering Karnataka, Kerala and Tamilnadu with production of about 100 metric tons (Kuruvilla et al. 2004).

Vanilla cultivation is severely hindered by the incidence of various diseases. It is susceptible to many fungal diseases such as foot rot and wilting which is caused by *Fusarium oxysporum* and *Phytophthora* sp., *Sclerotium* rot caused by *Sclerotium rolfsii*, leaf rot, blights and brown spots of anthracnose caused by *Colletotrichum gloeosporioides* (Divakaran et al., 2008). Disease suppression by biocontrol agents is the sustained manifestation of interactions among the plant, the pathogen, the biocontrol agent, the microbial community in and around the plant and the physical environment. Inducing a plant's own defense mechanism by prior application of a biological agent is an emerging concept and strategy in plant disease management. Plant growth promoting rhizobacteria were able to colonize plant internal tissues and improve

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plant growth by applying various mechanisms including biocontrol (Pleban et al., 1995, Shishido et al., 1999). The endophytic association may provide better advantage to the host plant than the rhizosphere interaction. Confocal laser scanning microscopy (CLSM) in combination with GFP is a powerful tool for examining plant- microbe interactions. Hence in this study, to establish the ability of the rhizosphere bacteria for endophytic colonisation, the selected bacteria were tagged with green fluorescent protein and were visualised in the plant tissue using confocal laser scanning microscopy.

Plant-microbe interactions can be studied in detail with the help of tracking methods that can facilitate the visualization and localization of microbes in plant tissues. The Green Fluorescent Protein (GFP) is widely used as a reporter in studies of gene expression and protein localization in diverse organisms. Bacterial cells tagged with GFP can be enumerated *in situ*, and samples do not need to be disturbed by techniques such as fixing, washing, hybridization or staining (Tombolini et al., 1998). GFP, a 27 kD polypeptide (Prasher et al., 1992) which converts the blue chemiluminescence of a Ca^{2+} sensitive photo protein Aequorin originally descrided from the jelly fish *Aequorea victoria* into green light has been proved to be a simple, attractive and excellent marker system for studying plant-microbe interaction (Chalfie et al., 1994).

The aim of this study was to establish the colonization ability of the potential biocontrol isolates in vanilla leaves and its efficiency to control phytopathogens of vanilla under green house conditions.

MATERIALS AND METHODS

Isolation of *Pseudomonas* sp.

Pseudomonas spp. were isolated from rhizosphere soil of vanilla plants by standard soil isolation method using King's B (KB) agar medium supplemented with nystatin. The inoculated plates were incubated at 30°C for 48 h. Colonies that came up on KB plates were observed under UV light on a transilluminator. The green fluorescent colonies under UV light were picked up, purified by repeated streaking on the same medium and its morphological and biochemical characters were studied. *P. fluorescens* strain MTCC 1748 obtained from Microbial Culture Collection Centre Chandigarh was used as reference strain.

In vitro screening of *Pseudomonas* spp. for their biocontrol activity

Ten predominant rhizobacteria isolated from different locations along with standard cultures of *P. fluorescens* were tested for their antagonist effect against three fungal pathogens viz. *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotium rolfsii* by dual culture method. Each fungal pathogen was grown on potato dextrose agar (PDA) plate. With the help of a sterile cork borer, a disc (6 mm) of fungal growth from this plate was taken and placed at one side of a fresh PDA plate. 24 h old culture of each bacterial strain was streaked parallel on the opposite side of the fungal disc 4 cm away from the disc. The plates were kept for incubation at 30°C for 96 h. Visual observations on the inhibition of hyphal growth of fungal pathogens were recorded after incubation and compared with the PDA plates simultaneously inoculated with the fungal pathogens alone.

Identification of selected rhizobacteria

The selected strains were subjected to cultural, morphological, and biochemical characterization as mentioned in Bergey's Manual of Determinative Bacteriology. 16SrDNA sequencing was done and the sequences were analyzed using the gapped BLASTn (www.ncbi.nlm.nig.gov) search algorithm. The sequence generated in this study was deposited in the NCBI GenBank database.

Mechanism of biocontrol activity

A simple plate assay was performed to find out the effect of volatile compounds produced by the isolates. Fungal culture discs (5mm) of *F. oxysporum, R. solani, S. rolfsii* were inoculated in the centre of PDA plates. The lid of fungal culture containing plates was removed and the base plate was immediately transferred to the top of the base plate containing the culture of biocontrol agents. It was then sealed tightly with parafilm and incubated for 7-8 days. Control plates with pathogen alone were also maintained. Reduction in hyphal growth of fungal pathogens was calculated.

Hydrogen cyanide production was assessed as per the method of Wei et al. (1991). All the antagonistic bacteria were inoculated in King's B medium and siderophore production was checked by $FeCl_3$ test (Neilands 1981). The isolates were grown on succinate medium to test the salicylic acid production. Chitinase enzyme activity was assayed spectrophotometrically by the method suggested by Boller and Mauch (1988) using colloidal chitin as substrate. The peroxidase activity was estimated using guaiacol as substrate (Hammerschmidt et al. 1982).

Evaluation of biocontrol potential of *Pseudomonas* sp. against fungal pathogens of Vanilla under green house condition

A pot culture experiment was conducted to assess biocontrol potential of the isolates *P. fluorescens* and *P. putida* against fungal pathogens of vanilla by coinoculation of the pathogens and biocontrol agents (Ganesan and Gnanamanickam, 1987). For inoculum preparation, the antagonistic bacterial isolates were grown separately in Tryptic Soy Broth (TSB) and incubated at 28°C for three days. Bacterial cells were harvested by centrifugation (7,000 rpm for 20 min). The pellet was washed in sterile distilled water and cells were then resuspended in sterile saline solution. The cell density was adjusted to get approximately 8×10^9 CFU ml^{-1} (Van et al., 2000).

Sporulated pure cultures of the isolated fungal pathogens, prepared on PDA medium were selected for the preparation of spore suspensions of each fungal isolate. A total volume of 20 ml sterile water was spread in aliquots on a culture plate and the fungal colony surface was lightly scraped using a sterile spreader. The cultures were filtered through Whatman No. 42 filter paper in to a sterile glass bottle. Spore counts were taken using a haemocytometer and the suspension was adjusted to have approximately 1×10^7 spores/ml.

Three nodded vanilla cuttings were planted in unsterilized soil, sand and cow dung mixture in 1:1:1 ratio. The potting mixture was filled in 6 x 8 inch polybags (1.5 kg/bag). The potting mixture without inoculation served as control. The soil had a pH of 7.6,

Table 1. *In vitro* screening of *Pseuodomonas* sp. against Phytopathogens of vanilla (percentage of inhibition after 5 days).

Biocontrol agent	*Fusarium oxysporum*	*Rhizoctonia solani*	*Sclerotium rolfsii*
P1	38.56 ± 0.24	43.72 ± 0.12	48.62 ± 0.29
P2	46.74 ± 0.3	44.47 ± 0.47	22.39 ± 0.48
P3	36.28 ± 0.5	36.44 ± 0.39	47.70 ± 0.33
P4	55.27 ± 0.47	57.68 ± 0.31	48.61 ± 0.42
P5	43.52 ± 0.25	49.73 ± 0.43	48.60 ± 0.47
P6	50.89 ± 0.66	54.51 ± 0.38	50.11 ± 0.19
P7	60.93 ± 0.21	58.52 ± 0.48	54.38 ± 0.26
P8	49.12 ± 0.15	56.40 ± 0.24	46.49 ± 0.24
P9	45.39 ± 0.12	42.75 ± 0.46	38.38 ± 0.24
P10	39.91 ± 0.24	34.22 ± 0.34	36.54 ± 0.36

Values are average of three replications. Results represented as Mean ± SD

0.18% organic carbon, 0.06% of available nitrogen, 0.006% of available phosphorous and 0.012% potassium. The soil had bacterial population of 3.7x 10^5 cfu/g, fungi of 3.98x 10^3 cfu/g and actinomycetes of 1.67x10^3 cfu/g. The plants were watered twice a day to maintain optimum soil moisture regime and kept under greenhouse condition with ambient irradiance, temperature and air humidity. The pots were arranged in a completely randomised design with three replications.

Vanilla cuttings were artificially inoculated with bacterial cell suspensions in sterile saline solution (8 x 10^9 CFU ml^{-1}) by foliar spray. Two such sprays were given in week interval as pre inoculation. One week after pre inoculations, the pathogen was given. Seven days after inoculation symptoms appeared as small lesion around the pinpricks. The appearance of visible symptoms was recorded and the severity was measured as the total number of leaves infected in all plants in each treatment. The plants were allowed to grow up to 60 days and observation on the percent leaves infection was calculated by using the formula:

$$\text{Percentage of leaves infection} = \frac{\text{Number of leaves infected}}{\text{Total number of leaves}} \times 100$$

Transformation of the endophyte

A portion of the freshly streaked bacterial culture was transferred to 1.5 ml of pre warmed C-medium using an inoculation loop. The cells were suspended by gently mixing and the tubes were incubated at 37°C for 2 h in a shaker. The bacterial cells were pelleted by centrifugation for 1 min. The cells were resuspended in 300 µl of T solution and incubated on ice for 5 min. It was centrifuged for 1 min in a micro centrifuge and the supernatant was discarded. The pelleted cells were resuspended in 120 µl T solution and incubated 5 min on ice. 2.5 µl of the ligation mixture containing 14 ng vector DNA (pGFPuv) was taken in a new microcentrifuge tube. It was chilled on ice for 2 min. 50 µl of the prepared cells was added to the tube containing the vector DNA and incubated for 5 min on ice. The cells were plated immediately on pre warmed LB-ampicillin plate (100µg/ml) and incubated overnight at 37°C. The regenerated colonies were subcultured and viewed under a UV transilluminator.

Spraying of the gfp-tagged endophytes and its colonization

Transformed colony was inoculated into TSB and incubated for 48 h to reach cell density of 1x 10^8 cells/ml. The broth culture was diluted 1/5 using sterile distilled water and sprayed on the leaves and petioles of the pot cultured cuttings of Vanilla twice with an interval of 4 days. The cuttings were uprooted and the leaves and petioles were surface sterilized using 2% sodium hypochlorite. Cross sections of the petiole was prepared and examined for fluorescence under the confocal laser scanning microscope (Leica confocal system).

The data were subjected to statistical analysis by 'F' test and the critical difference was calculated by student's 't' test at 0.05 P level of significance (Linu et al., 2009).

RESULTS

Isolation and Screening of *Pseudomonas* sp. against phytopathogens

All the 10 rhizobacterial isolates of *Pseudomonas* sp. were tested for their biocontrol potential against three fungal pathogens and the results are presented in Table 1. The *Pseudomonas* sp. isolate P7 showed maximum inhibition against *F. oxysporum* (60.93 ± 0.21%) *R. solani* (58.52 ± 0.48 %) and *S. rolfsii* (54.38 ± 0.26 %). The isolate P4 showed 55.27 ±0.4% inhibition against *F. oxysporum*, 57.68 ±0.31% against *R. hizoctonia solani* and 48.61 ± 0.42% against *S. rolfsii.* These two isolates were selected for further studies.

Identification of *Pseudomonas* sp.

The selected isolates were was gram negative motile rods. The organism showed citrate utilization and nitrate reduction. Catalase and oxidase tests were positive. Based on the biochemical reaction, the isolate P4 was identified as

Table 2. Growth reduction of fungal pathogens due to the organic acids produced by the isolates *P.putida* (P4), *P. fluorescens* (P7) and standard strain *P. fluorescens* MTCC 1748.

Type of organic acid	Fungal pathogens	Percentage (%) of growth reduction		
		P4	P7	Standard
Volatile organic acid production by the isolate	*Fusarium oxysporum*	34.48±0.6	89.31±0.26	58.46±0.22
	Rhizoctonia solani	31.79±0.21	73.15 ±0.13	68.45±0.12
	Sclerotuim rolfsii	31.10±.009	56.47 ± 0.41	51.83±0.09
Non volatile organic acid production	*Fusarium oxysporum*	34.68±0.08	80.43 ± 0.32	28.45±0.22
	Rhizoctonia solani	31.85±0.15	80.33 ± 0.23	78.49±0.10
	Sclerotuim rolfsii	31.01±1.02	69.31 ± 0.23	51.48±0.42

Values are average of three replications. Results represented as Mean ± SD.

Pseudomonas putida and the isolate P7 was identified as *Pseudomonas fluorescens*.

Results of BLAST search of 16S rDNA sequences of the isolate P4 showed close similarity with *P. putida* and the isolate P7 showed close similarity with *P. fluorescens*. The sequences of the isolated organisms were deposited in the NCBI gene bank and culture collection centre and got the accession number JF701675 for *P. putida* (P4) and JN578642 for the isolate *P. fluorescens* (P7).

Mechanism of biocontrol activity

The two selected biocontrol agents and the standard reference strain P*. fluorescens* (MTCC 1748) were capable of producing volatile compounds and reduced the growth of the pathogens. *P. fluorescens* showed greater inhibition against *R. solani* (80.11%). The two isolates P4 and P7 were found to produce more non volatile organic compounds than the reference strains against *F. oxysporum* (Table 2).

The isolates were found to produce HCN, siderophore and salicylic acid. The isolate P7 showed higher siderophore production compared to the isolate P4 and standard culture. In the case of salicylic acid production, the isolate P7 produced 0.129 mg of salicylic acid per 50 ml of culture whereas the isolate P4 recorded 0.089 mg of salicylic acid production per 50 ml of culture.

Chitinase enzyme activity was assayed spectrophotometricaly at 585 nm. The enzyme activity of *P. fluorescens* treated plant was 3.5 µglcNAc/min/g tissue and control (non treated plant) was 1.5 µg lcNAc/min/g tissue. The peroxidase activity of *P. fluorescens* was estimated as 160 units/l and control showed 100 units/l after 45 days.

Evaluation of biocontrol potential of isolates against fungal pathogens of vanilla under green house condition

Visible symptoms started to appear from the fifth day after inoculation with *F. oxysporum, R. solani* and *S. rolfsii* in the respective control plants. The symptoms were in the form of leaf yellowing which later turned to leaf rotting. The rotting extended to leaf sheath and rarely to the pseudo stem also. Observations were recorded in terms of number of leaves infected and the severity was recorded as the total number of leaves infected in all plants in each treatment. In control plants inoculated with *F. oxysporum*, *R. solani,* and *S. clerotium rolfsii* alone, the infection rate was very high and severity was near 90%. Besides leaf yellowing and leaf rotting, root rotting followed by wilting and dying of seedlings were also noticed. In all cases, where bioagents were inoculated first and later cross inoculated with phytopathogens, disease symptoms were not visible even after 15 to 20 days after inoculation (Table 3). The isolated *P. fluorescens* was found to be an efficient biocontrol agent compared to *P. putida.*

Transformation of *Pseudomonas* with pGFPuv

The antagonistic endophyte P7 which was identified as *P. fluorescens* was successfully transformed with pGFPuv. The transformed colonies in the LB-Ampicillin plate exhibited green fluorescence when viewed in UV light (Figure 1).

The transformed colonies were sprayed onto the leaves of the pot cultured vanilla and colonization of the isolate was confirmed by the presence of fluorescent bacteria inside the tissue when cross sections of the petiole were examined under confocal laser scanning microscope (Figure 2a and 2b). The bacteria were found to be localized within the intercellular spaces of the tissue

DISCUSSION

Microbial antagonists used in this study were isolated from rhizosphere soils of Vanilla. It has been suggested that microorganisms isolated from the rooted rhizosphere of a specific crop may be better adapted to that crop and may provide better control of diseases than organisms originally isolated from other plant species. Such plant

Table 3. Evaluation of phytopathogens against microbial antagonists in vanilla plants Pre inoculation with biocontrol agent.

Treatment	Pre inoculation with bio- control agents	Cross inoculation with pathogen	Percentage of leaves infection*
T1	*P.flourescens*	*Fusarium oxysporum*	7.18
T2	*P.flourescens*	*Rhizoctonia solani*	10.31
T3	*P. flourescens*	*Sclerotum rolfsii*	11.31
T4	*P.putida*	*Fusarium oxysporum*	20.74
T5	*P. putida*	*Rhizoctonia solani*	30.54
T6	*P. putida*	*Sclerotum rolfsii*	39.17
T7	Control (no biocontrol agent)	*Fusarium oxysporum*	90.27
T8	Control	*Rhizoctonia solani*	93.26
T9	Control	*Sclerotum rolfsii*	88.40

CD (5%) =1.95. Values are mean of three replicates.

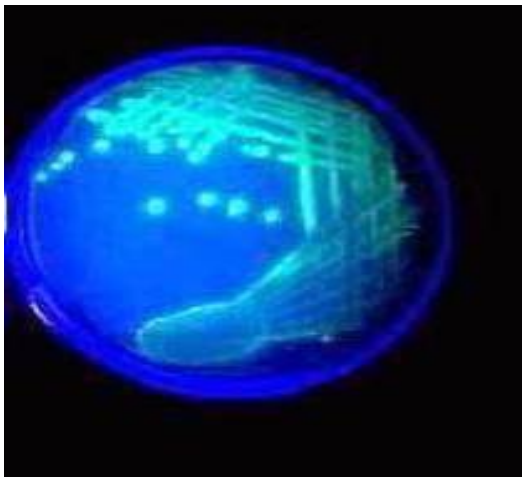


Figure 1. Transformed colonies, in the cross section of leaf, exhibiting green fluorescence.

associated microorganisms may prove to be better bio-control agent because they are adapted to rhizosphere effect of particular plant (Srivastava et al., 2010).

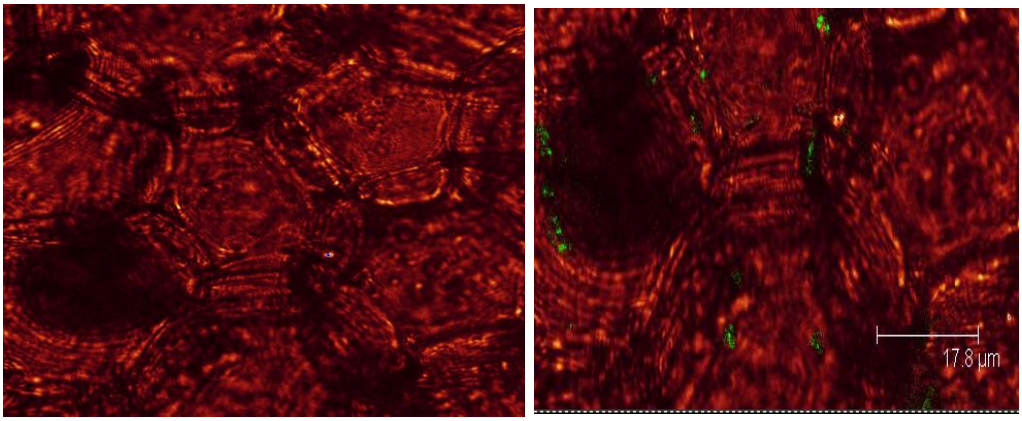
P. fluorescens effectively suppressed the disease incidence in vanilla. These results are in line with those of Ganesan and Gnanamanickam (1987) who recorded 99% protection of groundnut plants from *S. rolfsii* infection when they were inoculated with the native strain of *P. fluorescence* under green house conditions. Bhatia et al. (2005) also noticed significant reduction in the incidence of collar rot of sunflower in *S. rolfsii* infested soil due to seed bacteriazation with florescent pseudo-monades.

P. fluorescence are known to inhibit the growth of plant pathogens by diverse mechanisms such as antibiotic production (Hill et al., 1994), siderophore production (Loper, 1988); HCN release (Voisard et al., 1989) and lytic enzyme release (Fridlender et al., 1993). Hence they have been advocated as ideal biocontrol agents and plant growth promoting rhizobacteria. Induction of resistance by *P. fluorescens* is an additional mechanism by which these bacteria protect several crop plants against pests and diseases (Anand et al., 2010). *P. fluorescens* used in this study were also found to produce volatile and non volatile compounds, siderophore, HCN, salicylic acid, peroxidase and chitinase enzymes which are attributed to their biocontrol efficiency

The study of localization, pattern of colonization and survival of endophytic bacteria within the plant tissue is an indispensible area of research in a scenario where endophytes have been used as biocontrol agents. The failure of many biocontrol agents is usually attributed to their inefficiency to colonize the interior of the plant .The present study employed the use of the antibiotic, rifampicin and the auto fluorescent protein, GFP to study the colonization of antagonistic endophytes. Rifampicin resistance is the most commonly used marker to study population dynamics and survival of plant growth promoting and disease suppressing *Pseudomonas* sp. after their introduction in plants (Bahme and Schroth, 1987).

In the present study, the most effective biocontrol agent *P. fluorescent* P7, which was isolated from vanilla crop, was used for transformation with pGFPuv vector. The strength of GFP as a marker lies in the detection of individual cells in a nondestructive manner. GFP expressing plasmids can be used to simplify the detection and locate the position of an individual cell on plant roots, as reported by Bloemberg et al. (1997) in their study where they observed GFP–containing cells of *P. fluorescens* WCS365.

Lagopodi et al. (2002) reported that in the interactions between the *Pseudomonas* biocontrol sp. (tagged with RFP) and the fungal pathogen, *F. oxysporum* (tagged with GFP); both competed for the colonization of the same niches and directly interacting each other. At sites were bacteria were present, infection of the root by penetration of *Fusarium* was not observed. Bloemberg et al. (1997) proved that inside the plant tissue, endophytic



a b

Figure 2a. Presence of Green fluorescence protein (*gfp*) - tagged bacteria in the cross sections of petiole. A, Control; 2b, *gfp* - tagged bacteria in the cross sections of petiole.

bacteria either remain localized in a specific plant tissue like the root cortex or colonize the plant systematically by transport through the conducting elements or apoplast.

This is in agreement with present study where the marked antagonistic Pseudomonas species were seen with in the vanilla leaf tissues. Advantages of the use of GFP in comparison with other reporters or dyes is that GFP is present within the cell as a product of gene expression and that the visualization does not require any fixation or preparation protocols, which may influence cellular pro-perties. Furthermore it does not require substrates or additional energy such as often in the case of biolumi-nescence. Another possibility of the use of auto fluore-scent proteins involve dual imaging where the fungal pathogen and the bacterial endophyte are tagged with different auto fluorescent proteins so that their interact-tions can be observed well.

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Full Length Research Paper

Occurrence of multiple antibiotic resistance phenotype and class 1 integron in bacteria isolated from diabetic foot ulcers

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Resistance profile to antibiotics and the occurrence of class-1 integron in 23 bacteria isolated from diabetic foot ulcers (DFUs) were analyzed. Among 23 isolates, 16 elicited resistance to at least two antibiotics and seven were sensitive. Majority of the isolates were resistant to spectinomycin and streptomycin (68.75%) followed by trimethoprim (62.50%) and ampicillin (50%). Among the antibiotics resistant isolates, six exhibited resistance to four different groups of antibiotics and one showed resistance to 11 antibiotics (six antibiotic groups). 17 bacterial strains possessed class-1 integrons with an amplicon size of 0.30 to 2.40 kb which suggested their role in conferring resistance to antibiotics. 2-D gel electrophoresis of proteins extracted from *Escherichia coli* DF39TA grown with antibiotics revealed significant alteration in total proteome as compared to control culture. Five spots showing four fold up regulation were identified by MALDI TOF MS as OmpX, OmpA, OmpA-OmpF, Omp-tolC and chaperone protein DnaK. Changes in abundance of above proteins following growth with antibiotics may be beneficial for antibiotic resistant bacteria to adapt under adverse environmental conditions. Findings of this study suggest that infection with multiple antibiotic-resistant bacteria is common to DFUs and resistance is mediated by class-1 integrons.

Key words: Diabetic foot ulcer, antibiotic resistance, polymerase chain reaction (PCR), class-I integron, 2-D gel electrophoresis, *Escherichia coli*.

INTRODUCTION

Diabetic foot infection is a common and potentially devastating complication that can progress rapidly to irreversible septic gangrene necessitating amputation of the foot. All diabetic foot ulcers (DFUs) are contaminated with a variety of organisms which are aerobic Gram-negative bacilli, anaerobes, and certain Gram-positive species (Gadepalli et al., 2006; Singh et al. 2009). The specific organisms found in diabetic foot infections differ not only from patient to patient, but also from one part of the country to another (Ozer et al., 2010). Individuals with diabetes have at least a 10-fold greater risk of being hospitalized for soft tissue and bone infections of the foot

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Abbreviations: DFUs, Diabetic foot ulcers; **MDRO,** multidrug resistant organisms; **MDR,** multi drug resistance; **PCR,** polymerase chain reaction; **MR,** methyl red; **NCBI,** National Center for Biotechnology Information; **TCA,** trichloro acetic acid; **IEF,** isoelectric focusing; **SDS-PAGE,** sodium dodecyl sulphate-polyacrylamide gel electrophoresis; **MIRSA,** methicillin resistant *Staphylococcus aureus*; **ESBL,** extended-spectrum β-lactamase.

than the individuals without diabetes (Margolis el al., 2002). Approximately 50% of patients that fail antibiotic treatment will eventually require amputation.

Diabetic patients with foot ulcers have several factors that may be associated with a high risk of multidrug resistant micro-organisms (MDRM) carriage, such as inappropriate anti-biotic treatment, chronic course of the wound, reduced antibiotic concentration in the tissue and reduced antimi-crobial effect in the wound environment and frequent hospital admission (Hartemann-Heurtier et al., 2004; Gadepalli et al., 2006; Ozer et al., 2010). The most important cause of antimicrobial resistance is overuse or inappropriate use of antibiotics (Margolis el al, 2002). It is also known that peripheral arterial diseases are often present in patients with DFUs and may lead to poor penetration of antibiotics into the lower limb tissues, thereby promoting growth of resistant bacterial strains. The widespread incidence of multi drug resistance (MDR) in both clinical and environmental settings is a potential threat to public health in most parts of the world (Saenz et al., 2004; Baker-Austin et al., 2008; Qing et al., 2012). Several reports suggest that inadequate selection and abuse of antimicrobials may lead to the development of resistance in various other bacteria and make the treatment of bacterial infections more difficult (Kolar et al., 2001).

It was a general belief that plasmids and transposons facilitate the spread of genetic material between species or genera of bacteria (Kolar et al., 2001; Harbottle et al., 2006). However in the 1980s, genetic elements integrons were identified on these mobile elements (Stokes and Hall, 1989). During the last few years the integron/gene cassette system has emerged as one of the best examples of capture and expression of new genes (Harbottle et al., 2006), and became a hot topic of research for understanding the mechanism of antibiotic resistance (Bzrlow and Pemberton, 2004). Integrons possess two conserved segments, the 5' CS and the 3' CS, separated by a variable region (VR), which include integrated antibiotic resistance genes (Qing et al., 2012). The 5' CS contains the *int* gene, a gene cassette insertion site, *att*I and, on the opposite strand, a common promoter region, P1-P2, directed towards the site of integration. Most inserted cassettes lack their own promoter and are expressed from the common promoter region. Integrons are classified according to the integrase sequence (El-Najjar et al., 2010).

Till now, five classes of integrons (classes 1, 2, 3 4, 5) have been reported (Mazel, 2006). Of these, class 1 integrons are most common among multi-drug resistant Gram-negative genera including *Escherichia* (Saenz et al., 2004), *Klebsiella* (Girlich et al., 2000), *Salmonella* (Gebreyes and Altier, 2002), and *Serratia* species (Centron and Roy, 2002).

Polymerase chain reaction (PCR) amplification across highly conserved sequences has proved very useful and routine tool for screening and characterization of integrons from different bacteria (White et al., 2001; Chang et et al., 2009). Although increasing incidence of antimicrobial resistance is becoming a serious problem in India, there is paucity of data dealing with the incidence of multiple-antibiotic resistance bacteria from DFUs. Additionally, little is known about the protein turn over in integron-possessing bacteria following growth with antibiotics. The aim of this study was to determine antibio-tic susceptibility profile and screening of class 1 integron in bacteria isolated from DFUs. An attempt was also made to study alteration in protein profile (proteome) of a multiple-antibiotic resistant bacterium following growth with a combination of antibiotics.

MATERIALS AND METHODS

Patients

This study was conducted in the Department of Endocrinology and Metabolism, and Department of General Surgery, Sir Sunderlal Hospital, Institute of Medical Sciences, in collaboration with the School of Biotechnology, Banaras Hindu University, Varanasi. The study was conducted after seeking prior approval of the ethical committee of the Institute (Ref. No. Dean/2009-10/555 dated July 11, 2009). In total, 116 diabetic patients attending to the hospital between January 2010 and October 2011 were examined and four patients suffering from severe DFUs (grades IV and V) were selected for this study. Prior written consent was obtained from every recruited patient. Grading of DFUs that is, grade 0-hyperkeratosis; grade I -superficial ulcers; grade- II deep ulcers; grade- III tendonitis, osteomyelitis, cellulites, or abscess; grade- IV gangrene of a toe or forefoot; and grade -V massive gangrene of the whole foot was done according to Wagner (1981).

Sample collection

Initially, debridement was done with meticulous care to eliminate the colonizing bacteria from the ulcers. For sample collection, each DFU was cleaned with sterile saline and thereafter superficial swab sample was collected from the center of ulcer by applying a sterile cotton-tipped applicator. Tissue biopsy samples were obtained from the deep tissue of the ulcer using a sterilized punch biopsy needle (6 mm) under local anaesthesia. All specimens were transported by sterile containers; swabs in a tube containing sterile saline and biopsy samples in containers without medium. The specimens were examined quickly in the microbiology laboratory for Gram's staining.

Isolation and identification of bacteria

Each sample (swab and biopsy) was plated directly on different aerobic growth media such as 5% sheep blood agar, MacConkey agar, and chocklate agar and the plates were incubated at 35°C in an incubator. Biopsy samples were gently macerated before inoculation. The plates were examined after 24-36 h of incubation and distinct colonies appearing on each plate were picked up and restreaked on respective media. Isolates with distinct morphotypes from each plate were selected for further characterization. Tentative identification of different isolates was made on the basis of Gram's staining and morphological characters as well as biochemical tests namely, catalase, nitrate reductase, urease, Simmons citrate utilization and methyl red (MR) as per the standard methods. Identity of six isolates which carried multiple antibiotic resistance character was confirmed by 16S rDNA amplification and sequencing.

Amplification and sequencing of 16S rDNA

Genomic DNA was extracted by DNeasy Tissue Kit (Qiagen, Germany) according to the instructions of manufacturer. 16S rRNA gene (1.5 kb) was amplified by universal primer in a final volume of 50 µl as described by Jha and Kumar (2009). Amplified products were subjected to electrophoresis in 1.0% agarose gel, stained with ethidium bromide and visualized under UV light in a gel documenttation unit (BioRad Laboratories, USA). The PCR amplified fragment was purified from the agarose gel using a QIAquick Gel Extraction Kit (Qiagen). Sequencing of amplified product was done on commercial basis from Chromous Biotech Pvt. Ltd. (Bangalore, India).

The sequences obtained were matched with the GenBank data base applying the algorithm BLASTn program to identify the most similar 16S rDNA (Altschul et al., 1997). The nucleotide sequences of the 16S rDNA of 6 bacterial species were submitted to National Center for Biotechnology Information (NCBI) GenBank and accession numbers have been obtained (Accession numbers, HQ163790, HQ163792, HQ163793, HQ163794, HQ163797, and HQ163798).

Antibiotic susceptibility test

Antimicrobial susceptibility test of different strains was done by the disc diffusion method using the Kirby-Bauer method (Bauer et al., 1966). Thirteen antibiotics; spectinomycin (100 µg), streptomycin (20 µg), trimethoprim (20 µg), gentamicin (10 µg), ampicillin (10 µg), tetracycline (30 µg), kanamycin (30µg), amikacin (30 µg), augmentin (30µg), cefoperazone (75 µg) meropenem (10 µg), piperacillin/-tazobactam (100/10 µg) and clindamycin (2 µg), were selected according to published recommendations and their widespread use in treatment of various diseases (Gadepalli et al., 2006). Ampicillin, penicillin, augmentin and clindamycin are penicillins, spectinomycin, streptomycin, gentamicin, kanamycin and amikacin are aminoglycosides, tetracycline belongs to tetracycline group, meropenem is a carbapenems, cefoperazone is a cephalosporin, and trimethoprim belongs to the group of chemotherapeutic agents. The disks were purchased from Micro Master Laboratories (Mumbai, India). Interpretation of result is based according to Clinical and Laboratory Standards Institute (CLSI) guidelines 2007. Antibiotic susceptible, intermediately susceptible or resistant ability were assessed by measuring the diameter (mm) of the clear zone around the disc. Isolates showing resistance to more than two groups of antibiotics were designated as MDR.

Amplification of class 1 integron

The class 1 integron was amplified using forward primer 5'-GGC ATC CAA GCA GCA AG-3', and the reverse primer 3'CS-5'-AAG CAG ACT TGA CCT GA-3' (Levesque et al., 1995), in a PTC-100 Thermal Cycler (MJ Research, Inc., USA). Reaction was performed in a final volume of 50 µl, which included 1.5 U of *Taq* DNA polymerase, 1 X *Taq* assay buffer containing 1.5 mM $MgCl_2$, 125 µmol of each dNTPs (Bangalore Genei, Bangalore), 50 pmol of each primers (Integrated DNA Technologies, USA) and 100 ng template DNA. Thermal cycle for the amplification was set at: initial denaturation at 94°C for 7 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, extension at 72°C for 2 min 30 s followed by final extension at 72°C for 7 min. 5 µl of the amplified PCR products were electrophoresed on a 1% agarose gel containing 0.5 µg/ml ethidium bromide.

Two-dimensional gel electrophoresis

Protein extraction was done according to dos Santos et al. (2010). Briefly, *E. coli* DF39TA cultures grown without antibiotics or with antibiotics namely tetracycline, kanamycin, spectinomycin and ampicillin (100 µg/ml each) were harvested by centrifugation at 12000 rpm for 15 min at 4°C. The cell pellet was washed three times with MilliQ water and suspended in 2.5 ml of buffer [10mM Tris-Cl, 20 mM DTT and 1mM phenylmethylsulfonyl fluoride (Sigma, USA), pH-7.8] and passed through intermittent sonication (Branson Sonifier, USA) at maximum output and duty cycle for 2-3 min at 4°C. The homogenate was centrifuged at 12,000 rpm for 20 min at 4°C. Cell extracts were treated with 1% SDS and subjected to trichloro acetic acid (TCA)-acetone precipitation. Precipitate was collected by centrifugation at 15000 rpm for 30 min at 4°C. The protein pellet obtained was washed twice with 90% acetone and thereafter air dried. To the dried pellet, 500 µl of rehydration buffer [8M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) IPG buffer (GE Healthcare Ltd., UK) and 0.3% (w/v) DTT] were added and the suspension was kept at 4°C for 8-10h with intermittent vortexing to solublize the protein pellet.

Isoelectric focusing (IEF) was carried out following the method of Gorg et al. (2000). Approximately 300 µg of protein was applied on immobiline dry strip (pH 4-7, 13 cm, GE Healthcare Bio-Sciences AB, Uppsala) and rehydrated overnight at 20°C with rehydration buffer. Strips were focused on Ettan IPGphor3 system (GE Healthcare Ltd., UK) at 20°C using the following seven step program: a) 0-200 V for 4 h; b) 200-500 V for 2 h ; c) 500-1000V for 2 h; d) 1000-2000V for 3 h; e) 2000-3500V for 2 h ; f) 3500-5500 V for 2 h (steps a-f in linear mode), and g) 6500V constant until 60 kVh reached. The current limit was set at 50 µA per strip. After IEF, each strip was incubated for 15 min in 10 ml of 50 mM Tris HCl buffer, pH 8.8, 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% BPB, and 125 mM DTT, followed by a second incubation step in the same buffer, excluding DTT, which was replaced by 125 mM iodoacetamide. Proteins were separated in second dimension on 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a vertical electrophoresis dual gel unit (Bangalore Genei, India) at constant current of 20 mA for 12 h and gels were stained with PlusOne Silver Staining Kit (GE Healthcare Ltd., UK) according to the instructions of manufacturer. 2-D gels were analysed using PDQuest 2-D analysis software (version 8.0.1) (BioRad Laboratories, USA). Images were analysed using stepwise spot detection and spot matching followed by differential expression analysis. Equal amount of protein was loaded in each gel and experiments were repeated at least three times.

Protein spot picking and mass spectroscopic analysis

Five distinct spots showing ca 4-fold increase in expression were excised from the gel by manual picking using sterile one touch spot picker into separate microcentrifuge tubes. Protein spots were sent to the Centre for Genomic Application (TCGA), New Delhi, India for matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectroscopic analysis. The proteins were identified by comparing peptide mass fingerprints at the NCBInr database using the Mascot search engine (http://www.matrixscience.com). The search parameters allowed for oxidation of methionines, carbamidomethylation of cysteines, one mis-cleavage of trypsin, and 30 ppm mass accuracy. The proteins were identified based on the first ranking result and Mascot scores >74, which indicated that the hits were significant.

RESULTS

Isolation and initial characterization of bacteria

23 strains of aerobic bacteria (10 from swabs and 13

Table 1. Isolation of multiple-antibiotic resistant bacteria from diabetic foot ulcers.

Patients ID	Grade of DFU*	Total types of bacterial species+	Multiple-antibiotic resistant isolates#
DF5	IV	*Escherichia coli* DF5SA *Enterococcus* sp. DF5SB *Klebsiella pneumoniae* DF5SC *Staphylococcus haemolyticus* DF5TA *Alcaligenes* sp. DF5TB *Pseudomonas* sp. DF5TC	*Enterococcus* sp. DF5SB *Staphylococcus haemolyticus* DF5TA *Pseudomonas* sp. DF5TC
DF18	IV	*Pseudomonas stuzeri* DF18SA *Alcaligenes* sp. DF18SC *Escherichia coli* DF18TA *Staphylococcus aureus* DF18TB *Pseudomonas aeruginosa* DF18TC	*Alcaligenes* sp. DF18SC
DF39	V	*Enterococcus* sp. DF39SA *Alcaligenes* sp. DF39SB *Pseudomonas stuzeri* DF39SC *Escherichia coli* DF39TA *Staphylococcus aureus* DF39TB *Pseudomonas aeruginosa* DF39TC *Proteus* sp. DF39TD	*Escherichia coli* DF39TA
DF36	IV	*Acinetobactor* sp. DF36SA *Enterococcus* sp. DF36SB *Klebsiella pneumoniae* DF36TA *Escherichia coli* DF36TB *Alcaligenes* sp. DF36TC	*Alcaligenes* sp. DF36TC

*, Grading of diabetic foot ulcer (DFU) was done according to Wagner's system; +, identification of isolates based on distinct morphological characters and biochemical tests; #, isolates showing resistance to more than three types of antibiotics were identified by 16S rDNA sequencing; DF, diabetic foot; S, swab; T, tissue; A-C, different isolates of tissue or swab.

from tissues) belonging to 8 genera and 11 species from DFUs of four patients were successfully isolated (Table 1). Of the 23 isolates, 20 (86.95 %) belonged to Gram-negative and 3 (13.04 %) to Gram-positive group. Two isolates namely *Escherichia coli* and *Alcaligenes* sp. were common in DFUs of all the four subjects whereas *Proteus* sp. and *Acinetobactor* sp were present in DFUs of DF39 and DF36 respectively. Interestingly, as many as 7 strains of bacteria were isolated from DFU of one patient (DF39). Occurrence of bacterial types was observed routinely higher in tissue (56.52%) than swab samples (43.47%).

Antibiotic sensitivity test

Antibiotic sensitivity test revealed that 16 isolates were resistant to either streptomycin and spectinomycin or trimethoprim. However, six isolates namely *Enterococcus* sp. DF5SB, *Staphylococcus haemolyticus* DF5TA, *Escherichia coli* DF39TA, *Alcaligenes* sp. DF18SC, *Pseudomonas* sp. DF5TC, and *Alcaligenes* sp DF36TC showed resistance to more than three groups of antibiotics (Table 2).

Out of these, *Staphylococcus haemolyticus* DF5TA, *Pseudomonas* sp. DF5TC, *Enterococcus* sp. DF5SB and *Alcaligenes* sp. DF36TC showed resistance to four different groups of antibiotics. *Escherichia coli* DF39TA, and *Alcaligenes* sp. DF18SC, were found resistant to more than four groups of antibiotics. Based on antibiotics resistance profile, above six isolates could be designated as multidrug resistant bacteria (Table 2).

Identification of multiple drug resistance bacteria

All the bacterial isolates were identified on the basis of morphological characters and routine biochemical tests (Table 1). However, six isolates showing multiple antibiotics resistance character were identified by 16S rDNA (1.5kb) sequencing. Based on 16S rDNA (1.5kb) sequence homology, these isolates were identified as.

Table 2. Antibiotic resistance phenotype and size of class 1 integron of different isolates.

Isolate	Phenotype of resistance	Integron size (kb)
Staphylococcus haemolyticus DF5TA	Amp, Tmp, Tet, Aug, Mer	0.30
Alcaligenes sp. DF5TB	Spt, Str,	1.0
Pseudomonas sp. DF5TC	Spt, Str, Kan, Tmp, Gen, Aug, Cld, Mer	1.60
Escherichia coli DF5SA	-	-
Enterococcus sp. DF5SB	Spt, Str, Amp, Tet, Kan, Tmp, Gen, Aug	1.60
Klebsiella pneumoniae DF5SC	-	-
Escherichia coli DF18TA	Spt, Str	1.0
Staphylococcus aureus DF18TB	Amp, Tmp	0.72
Pseudomonas stuzeri DF18SA	-	-
Alcaligenes sp. DF18SB	Amp, Tmp	0.72
Pseudomonas aeruginosa DF18SC	Spt, Str, Kan, Tmp, Gen, Aug,Cef, Cld,Mer	1.20
Escherichia coli DF39TA	Spt, Str, Amp, Tet, Kan, Tmp, Gen, Aug, Cef, Cld,Mer	2.40
Staphylococcus aureus DF39TB	-	-
Pseudomonas aeruginosa DF39TC	Amp, Tmp	0.72
Proteus sp. DF39TD	Spt ,Str, Amp	1.0
Enterococcus sp. DF39SA	-	0.85
Alcaligenes sp. DF39SB	Spt, Str	0.72
Pseudomonas stuzeri DF39SC	Amp,Tmp	0.72
Klebsiella pneumoniae DF36TA	-	-
Escherichia coli DF36TB	Spt, Str, Gen	1.60
Alcaligenes sp. DF36TC	Spt, Str, Kan, Tmp, Gen,Aug, Mer	0.85
Acinetobactor sp. DF36SA	-	-
Enterococcus sp. DF36SB	Spt, Str, Gen	1.60

Amp, Ampicillin; Amk, amikacin; Aug, augmentin; Cef, cefoperazone; Cld, clindamycin; Gen, gentamycin; Kan, kanamycin; Mer, meropenem; Pip/Taz, piperocillin/tazobactum; Spt, spectinomycin; Str, streptomycin; Tet, tetracycline and Tmp, trimethoprim.

Enterococcus sp DF5SB (HQ163798), *S. haemolyticus* DF5TA (HQ163797), *E. coli* DF39TA (HQ163793), *Alcaligenes* sp. DF18SC (HQ163792), *Pseudomonas* sp DF5TC (HQ163790), and *Alcaligenes* sp DF36TC (HQ163794), respectively.

Occurrence of class I integrons

Results of CS-PCR showed that 17 isolates harboured class 1 integron (variable region) with an amplicon size of ca. 0.3 to 2.4 kb (Table 2). It is evident from the result of Table 2 that five isolates had amplicon size of 0.72 kb, four had 1.60 kb, three contained 1.0 kb amplicon, two had 0.85 kb and the remaining three isolates had amplicon of 0.3, 1.20 and 2.4 kb size. Representation of amplified PCR product of class I integron of six isolates is presented in Figure 1.

Two-dimensional gel electrophoresis

Proteome analysis by 2-D gel electrophoresis of *E. coli* strain DF39TA grown with or without antibiotics showed distribution of protein spots in the region of pI 4-7. Figure 2 A-B depicts distribution of the protein spots of *E. coli* strain DF39TA grown without or with antibiotics (tetracycline, kanamycin, spectinomycin and ampicillin-100µg/ml each). Digital image analysis revealed the appearance of ca 200 and 266 spots in the untreated control and antibiotics-treated culture of *E. coli* strain DF39TA, respectively (Figure 2A-B). Of the 200 spots in the control culture, 126 spots matched with cultures grown with antibiotics. Analysis of spots of antibiotic-treated culture revealed that of the 126 spots, 62 were up regulated and 64 down regulated. It is also evident from the data of scatter diagram that 37 protein spots showed two-fold increase whereas 33 spots showed two-fold decrease in intensity following growth with antibiotics (Figure 2C). Several spots showed 3-4-fold increase or decrease in the intensity (Figure 2 D-E).

Five spots of interest (showing four-fold increase in intensity) were subjected to MALDI TOF MS for protein identification. Identity and other features of proteins are presented in Table 3. The five spots were identified as four unique proteins based on their possible function as OmpX, OmpA, OmpA-OmpF, Omp-tolC, and chaperone protein DnaK. Further analysis suggested that OmpX, OmpA, OmpA-OmpF, and Omp tolC are outer membrane

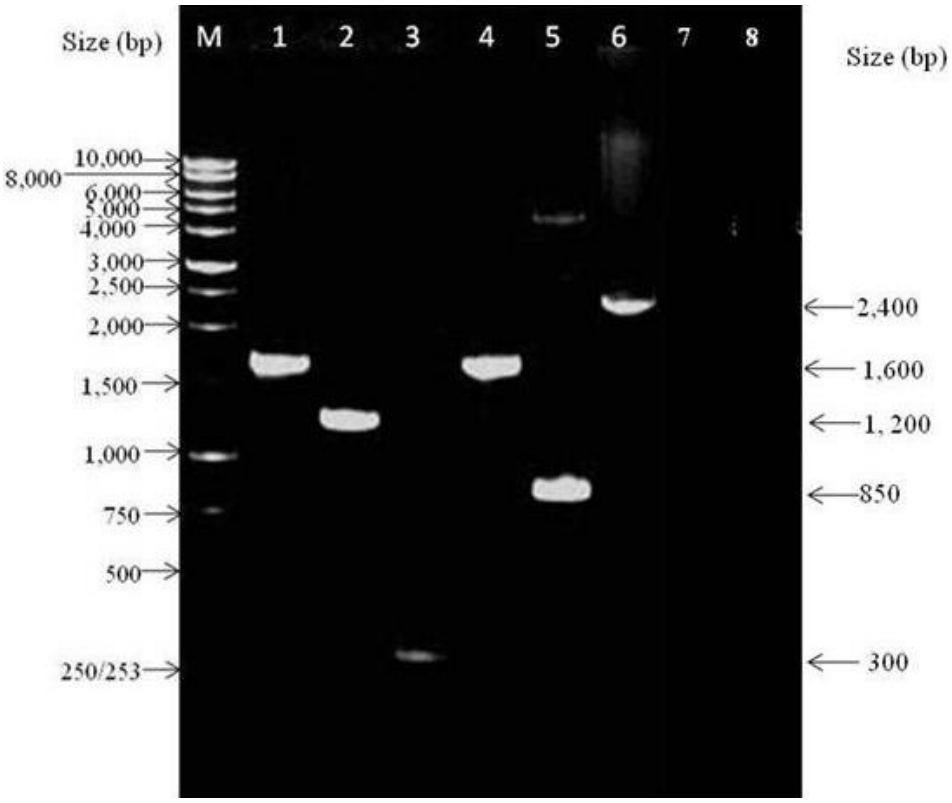

Figure 1. Polymerase chain reaction (PCR) amplification of class 1 integron with 5'CS-3'CS primers. Lane M, Molecular size marker (1 kb); lanes 1-8, various bacterial isolates; 1, DF5SB; 2, DF18SC; 3, DF5TA; 4,DF5TC; 5, DF36TC; 6, DF39TA; 7, negative control with *E. coli* JM109 template; and 8, negative control (without template).

(OM) proteins and chaperone DnaK is a cytoplasmic/-inner membrane protein (Table 4).

DISCUSSION

The present study suggests that the prevalence of multi drug resistance organism (MDRO) is fairly common in severe DFUs and supports the finding of earlier studies (Hartemann-Heurtier et al., 2004; Gadepalli et al., 2006). Hartemann-Heurtier et al. (2004) reported that about one-third of patients with a history of previous hospitalization for the same wound, and 25% of patients with osteo-myelitis, had MDRO-positive specimens. Gadepalli et al. (2006) also noted 44.7 and 56.0% ESBL-producing and methicillin resistant bacteria, respectively in DFUs from South India.

Incidence of high rate of antibiotic resistance in this study could be due to the fact that S.S. Hospital of Banaras Hindu University, Varanasi, is a tertiary care hospital with widespread usage of broad spectrum anti-biotics leading to selective survival advantage of bacteria. Additionally, increase in antimicrobial resistance might be the result of irrational use of antibiotics and the transfer of resistance genes by transport means including antibiotic resistant plasmids, bacterio-phages, transposons and integrons. Since a plasmid or transposon can carry seve-ral resistance indexes, simultaneous resistance to multi-ple antimicrobial agents may develop and the result would be MDROs. Although we could not estimate ESBL production in any isolates, the role of extended spectrum β-lactamase production in conferring resistance to anti-biotics may not be ruled out. The prevalence of methicillin resistant *Staphylococcus aureus* (MIRSA) and extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacteria in DFUs has been reported by other researchers (Hartemann-Heurtier et al., 2004; Gadepalli et al., 2006). An important finding of this study relates to the domi-nance of Gram-negative aerobic bacteria in DFUs. This is contrary to the findings of certain other workers who reported Gram-positive aerobes as predominant bacteria (Ozer et al., 2010).

Out of 16 isolates showing resistance to antibiotics, six isolates namely *E. coli* strain DF39TA, *S. haemolyticus* strain DF5TA, *Enterococcus* sp. DF5SB, *Pseudomonas* sp. DF5TC, *Alcaligenes* sp. DF18SC and *Alcaligenes* sp. DF36TC are of major concern as they eli-cited resistance to more than three groups of antibiotics.

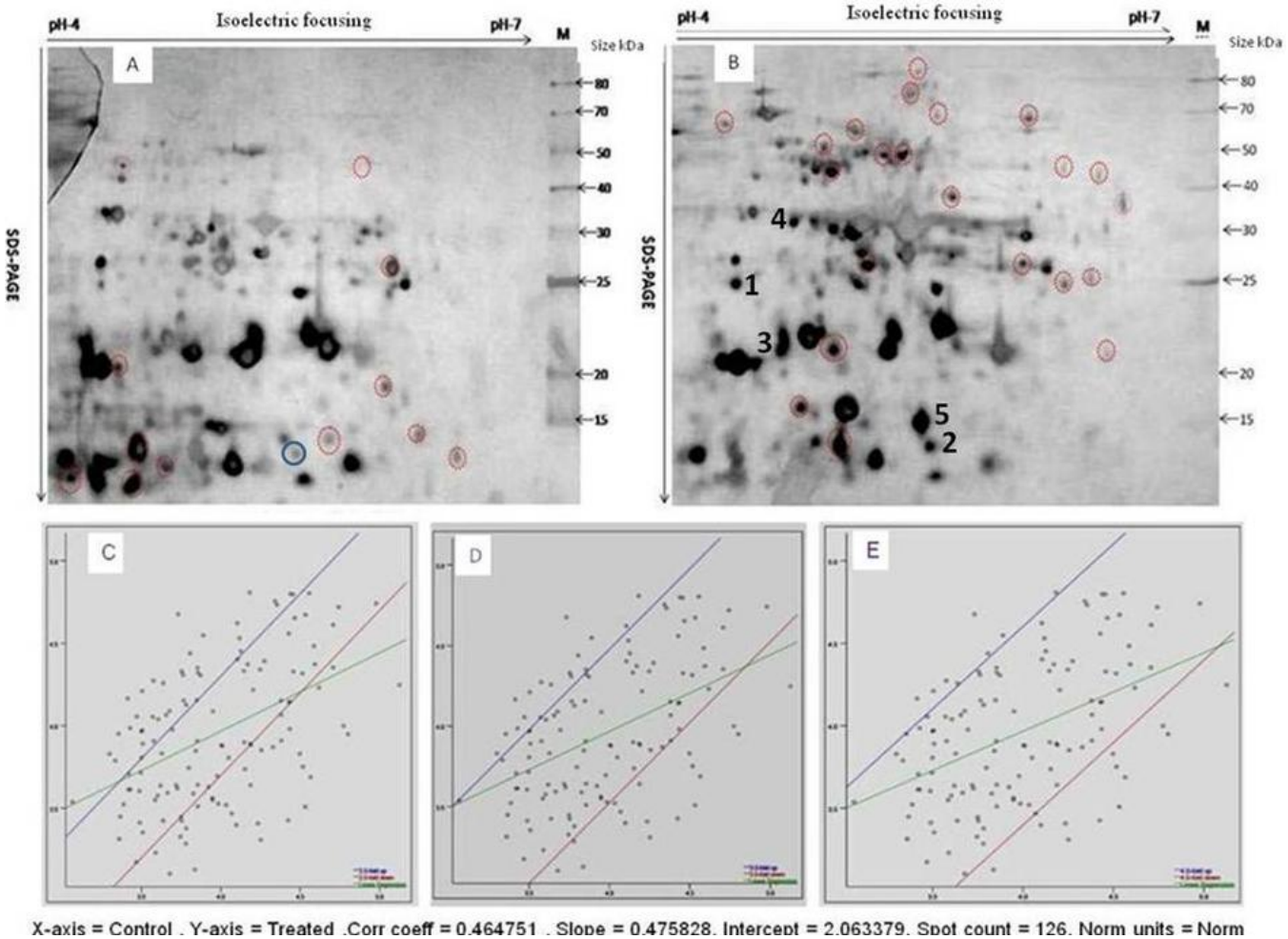

Figure 2. Two-dimensional gel images of protein spots of *E.* coli strain DF39TA. A, Control culture grown without antibiotic; B, *E.* coli strain DF39TA grown with four antibiotics namely ampicillin, kanamycin, tetracycline, and spectinomycin; C-E, scatter diagram of protein spots showing increase/decrease in intensity of spots: C, two-fold, D, three-fold, and E, four folds. X and Y axes represent spots of control and antibiotics-treated cultures, respectively.

Of the six isolates, *E. coli* strain DF39TA was resistant to 11 antibiotics belonging to six different groups. Findings of this study are close to that from non-pathogenic *E. coli* strains obtained from food products of animal origin and from fecal samples wherein multiple-antibiotic-resistant phenotypes to as many as 12 antibiotics of different groups were noted (Saenz et al., 2004). Additionally, clinical isolates namely *E. coli*, *S. haemolyticus, Enterococcus* sp., *Pseudomonas* sp., and *Alcaligenes* sp. have been reported to confer resistance to trimethoprim, streptomycin and spectinomycin (White et al., 2001; Lindstedt et al., 2003; Nogrady et al., 2005). All these reports suggest that infection with MDR strains may probably limit the choice of antibiotic treatment leading to longer duration of hospital stay and complica-ting the management of DFUs.

That the multiple-antibiotic-resistant phenotypes in various isolates could be due to the presence of class 1 integron of variable region (VR) is reinforced by the fact that 17 isolates showed amplification of amplicons in the range of 0.3 to 2.4 kb size. Our finding is in agreement with the data of El-Najjar et al. (2010) who reported prevalence of class 1 integron from uropathogenic *E. coli* strains resistant to antibiotics. As majority of the isolates (n-11) in this study showed resistance to spectinomycin, streptomycin and/or trimethoprim, it appears that the presence of aminoglycoside resistance (*aad*A) and trimethoprim resistance (*dfr*A) determinants play important role in growth and survival of bacteria. Above conclusion is also supported from the studies conducted in Asia and Europe where high prevalence of the aminogly-coside resistance determinant (*aad*A) and trimethoprim resistance determinant (*dfr*A) was noted in several isolates (Saenz et al., 2004; Gadepalli et al., 2006; Chang et al., 2009; El-Najjar et al., 2010).

To our knowledge, occurrence of classes 1 and 2 integrons in clinical isolates of *Enterococcus* spp. was not known earlier and has been reported only recently (Xu et

Table 3. Identification of up-regulated protein of *E. coli* strain DF39TA after growth with different types of antibiotics*.

Spot no.+	Protein	Gene ID	Protein accession No.	No. of amino acid	MW/ *pI*	Mascow score
1	OmpX	293403808	ZP_06648108.1	131	14759.2/ 5.75	200
2	OmpA	293409074	ZP_06652911.1	350	37479.1/ 5.78	587
3	OmpA-OmpF	293413461	ZP_06656886.1	350	37451.0/ 5.65	495
4	Omp tolC	293448943	ZP_06663796.1	490	53371.2/ 5.22	488
5	Chaperone protein DnaK	312965775	ZP_07780362.1	633	68522.3/ 4.81	395

*****Culture was grown with tetracycline, kanamycin, spectinomycin and ampicillin (100µg/ml each); +spots showing ca 4-fold up-regulation were selected for MALDI TOF MS analysis.

Table 4. Tentative function, localization, and gene ORF position of selected proteins of *E. coli* strain DF39TA after growth with antibiotics.

Protein	Gene ORF position	Trend in antibiotic treated vs. control	Biological process	Cellular location
OmpX	NZ_GG749217.1: 313566-313961	Four fold up regulated	Induced beta-lactam resistance	Outer membrane (OM)
OmpA	NZ_GG749328.1: 293351-294403	"	Stress survival	OM
OmpA-OmpF	NZ_GG749165.1: 897624-898676	"	Stress survival	OM
Omp tolC	NZ_GG749140.1: 467236-468708	"	Multidrug efflux and protein export	OM
Chaperone protein DnaK	NZ_ADUL01000057.1: 348814-350715	"	Stress Survival caused by antibiotics	Cytoplasm and inner membrane

al. 2010; Yan et al., 2010). Yan et al. (2010) were probably the first to report the presence of class 1 and 2 or both the integrons in clinical isolates of *Eneterococcus faecalis* and *Eneterococcus faecium.* Subsequently, Xu et al. (2010) also reported class 2 integron in clinical *E. faecalis* and class 1 integron in *E. faecium* from South China. Occurrence of integron in *Eneterococcus* spp in our study is new addition and strengthen the findings of above report. However, detailed characterization of *Eneterococcus* spp. integron is needed to find out resistance determinant present on this fragment.

Protein profile related to antibiotics resistance has been studied in a number of bacteria (Xu et al., 2006; Li et al., 2007; Roncada et al., 2009; dos Santos et al., 2010). However, little, if any, information is available pertaining to the changes in total protein profile of multiple antibiotic resistant bacteria from DFUs. Results of this study clearly suggest alteration both in qualitative and quantitative terms in proteome of *E. coli* strain DF39TA grown with or without antibiotics. Xu et al. (2006) employed proteomic approach to characterize functional outer membrane proteins of *E. coli* K-12 resistant to tetracycline and ampicillin. They identified outer membrane proteins such as TolC, OmpC and YhiU in this strain as a result of antibiotic resistance. Similarly, dos Santos et al. (2010) reported increase in abundance of 12 protein spots in *E. coli* resistant to piperacillin/tazobactum as compared to sensitive strain. Their study showed the role of a multidrug efflux pump system in *E. coli* resistance to piperacillin/tazobactum. We also noted more than four fold increase in expression of OmpX, OmpA, OmpA-OmpF, Omp-tolC, and chaperone protein DnaK in *E. coli* strain DF39TA under stress of antibiotics. Furthermore all these proteins excluding chaperone protein DnaK were identified as outer membrane proteins. It is well documented that these proteins are responsible for antimicrobial resistance in different bacteria (dos Santos et al., 2010).

Prediction of function of these proteins in our analysis also suggested their roles in mitigating stress or in inducing beta lactam resistance or in multidrug efflux pump system. Altogether our findings pertaining to protein turn over during antibiotics stress are consistent with earlier reports and it may be safely concluded that changes in protein abundance may allow multiple drug resistant microorganisms to develop molecular changes in an effort towards adaptation to adverse environmental conditions.

Conclusions

In conclusion, data obtained reveal the occurrence of multiple-antibiotic resistant bacteria in diabetic foot ulcers and suggest the role of class 1 integron in conferring resistance to antibiotics. The gene cassettes present in integrons may explain the phenomenon of broad resistance of these bacteria to a number of antibiotics. Our data also reveal subtle changes in the proteome of a multiple antibiotic resistant strain (*E. coli* strain DF39TA)

suggesting that the misuse of antibiotics may also interfere with cell physiology which in turn may pose problem in the treatment of infectious diseases. Since the worldwide prevalence of antibiotic-resistant bacteria is on increase and may cause serious threat to human health, understanding the mechanism (s) of antibiotics resistance using both genomics and proteomics approaches could assist in developing strategies for better treatment.

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Full Length Research Paper

Physiological and mutagenic effects of pathogenic and non-pathogenic bacteria on the last larval instar of *Bombyx mori* (Lepidoptera: Bombycidae)

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The present study investigated the physiological and genetical effects of non-pathogenic bacteria (G^-) *Escherichia coli* and entomopathogenic bacteria (G^+) *Bacillus thuringeinsis* on the mulberry silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). We used bioassay and molecular markers linked to 5th instar larvae to inject bacterial and analyze the response of *B. mori.* Also, the effect of both bacteria on the total and differential haemocytes count of the 5th instar larvae of *B. mori* was evaluated. Results revealed that injection with *E. coli* increased the total haemocytes counts (THCs) to about 59.09% of the control at 24 h post-infection. In this respect, *B. thuringeinsis* decreased the THCs by about 45.9, 58.39 and 69.4%, respectively, than control after 48 h post-infection at concentrations of 0.5, 1 and 1.5 ppm. Injection with *E. coli* increased the number of Pr, Pl, Gr and Oe. On the contrary, injection with *B. thuringeinsis* significantly decreased the number of Pr, Pl and Gr and Oe. The mutagenic effect of the bacterial injection produced and/or affected several proteins that killed or caused larval deformation through the alteration of the physiological and genetic processes.

Key words: *Bombyx mori*, haemocytes, corpora allata, DNA structure, *Escherichia coli, Bacillus thuringeinsis.*

INTRODUCTION

The silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) has been exploited as a silk producer in the silk industry for thousands of years. The recent success of transgenesis of the silkworm has opened new prospects for this insect species (Tamura et al., 2000). Silkworm diseases are the most important disease that inflect heavy loss to crops. Most losses in sericulture can be attributed directly to silkworm diseases. Among silkworm diseases, bacterial diseases are common, but in general, massive outbreaks are rare. Pathogenic bacteria of silkworm belong to a wide variety of genera, including *Bacillus*, *Enterobacter*, *Serratia*, *Aeromonas*, *Streptococcus*, *Pseudomonas* and *Staphylococcus* (Tao et al., 2011). However, when silkworms are physiologically weak, bacterial diseases can attack them, eliciting a heavy toll on sericulture (Aruga, 1994). The bacterial diseases affecting silkworm are called flacherie because the cadavers of silkworms that have died of these diseases lose elasticity, soften, and rot. Bacterial diseases of silkworms are usually only secondary to virus diseases. The chief diseases affecting silkworm

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is flasherie caused by *Bacillus* bacteria. One casual bacillus of silkworm flasherie is *Bacillus thuringeinsis* which is a widely distributed facultative entomologenous bacterium with as much as 34 varieties. It is a Gram positive spore formatting bacterium widely distributed in the soils of various regions of the world. The endotoxin produced by *B. thuringeinsis* is known to destroy the gut lining, causing paralysis and death in many insect species belonging to orders, Diptera and Lepidoptera including economically important insects in several kinds of silkworms (Aizawaza, 1971; Nataraju et al., 1991).

Insect innate immunity can be affected by juvenile hormone (JH) and 20-hydroxyecdysone (20E), but how innate immunity is developmentally regulated by these two hormones in insects and has not yet been elucidated. Fat body produces humoral response molecules and hence is considered as the major organ involved in innate immunity (Muramatsu et al., 2008). Tian et al. (2010) suggested that JH plays a positive role in the regulation of innate immunity in the larval fat body and the volume of Corpora allata (C.A.) was used as an indicator for its activity according to Pflugefelder (1948). Mulberry silkworm was selected as a model system for studying immunity responses against bacteria, because it is of great economic importance due to its unique white silk. Last larval instar of this silkworm is selected for the experiments; the main advantage is that in the last larval instar, the silk protein synthesis and most larval mortality was as a result of diseases caused in this stage.

In the present investigation, we studied the effect of some immunity responses of silkworm larvae such as, cellular immunity (total and differential haemocyte counts) against *E. coli* (G^-) and *B. thurengensis* (G^+) bacteria and also studied the effects of both *E. coli* and *B. thurengensis* at concentration of 1.5 ppm on the corpora allata (CA) activity during the last larval instar of *B. mori* to understand the effect of both bacteria on activation or inhibition of the CA activity and its relation with total and differential haemocytes counts.

MATERIALS AND METHODS

Laboratory animals

Silk worm, *B. mori* (PM X NB4D2) were reared on an artificial diet at 25 ± 2°C and RH 65 to 70%. Newly molted (day 0) fifth larval instar (weighing 1 to 1.5 g) was utilized for all experiments.

Preparation of bacteria

Non pathogenic bacteria (G^-) *E. coli* obtained from Biology Department, University of Taibah was grown in the room temperature for 24 h at 30°C on nutrient agar (3 g of beef extract, 5 g of peptone and 20 agar in 1 L distilled water), pH 7.0.

The commercial formulation of *Bacillus thuringeinsis (*Dipel®, Sumitomo Chemical Agro Europe (SCAE)) was taken from Applied Entomology Department, Faculty of Agriculture, Alexandria University. Three serial dilutions of the tested product (0.5, 1.0 and 1.5 ppm) of bacteria were used.

Bacterial injection

The newly molted 5th larval instar of *B. mori* were injected with *E. coli* (G^-) (1.1 x 10^6 cells/ml), *B. thuringiensis* (G^+) (Dipel) was injected at concentrations of 0.5, 1.0 and 1.5 ppm and physiological saline (Sigma, UK), as control. Each larva was injected with 50 µL in the dorsal segment of larvae by BD Micro-fine™ plus syringe. Five replicates, 60 newly molted larvae were used for each treatment.

Studies of haemocytes

For total haemocyte counts (THC), fresh haemolymph was collected after 24, 36 and 36 h of treatment and diluted 10- fold with a cold physiological saline buffer containing 0.4% trypan blue (Horohov and Dunn, 1983). Cells in the diluted haemolymph were counted using a Thoma haemocytometer (Essawy et al., 1999). Under phase contrast optics was as described by Arnold and Hinks (1976) after 24, 36 and 48 h. The THC was estimated according to the formula suggested by Jones (1962). The differential haemocyte counts (DHC) were estimated according to the technique used by Akai and Sato (1979) using fresh slid preparations.

Corpora allata activity

Corpora allata (CA) volume was used as an indicator of the juvenile hormone (JH) level (Pflugefelder 1948). Larvae were dissected every 24 h and until prepupal stage after treatment with *E. coli* (G^-), *B. thuringiensis* (G^+) at concentration of 1.5 ppm and control. The method of Armstrong and Carr (1964) was used to calculate the CA surface area.

Larval DNA studies

Genomic DNA was extracted according to the protocol described by Pither et al. (1989) with the following modification: Whole larvae homogenized in 100 µl of extraction buffer (1X PCR Buffer) 0.08% (w/v) of Tris base, 0.06% (w/v) of $MgC1_2$, and 0.02% (w/v) of $NH_4(SO_4)_2$ in 90 ml of HCl. The final volume was adjusted to 100 ml with distilled water. The ground larvae in solution were placed in a boiling water bath for 15 min. The tube is centrifuged at 6,000 rpm to get rid of debris. Larval tissues were centrifuged again at 6,000 rpm for 10 min at 4°C, resuspended in buffer (100 mM Tris-HCl (pH = 8.0), 100 mM EDTA, 250 mM NaCl). Sodium dodecyl sulfate and proteinase K were added to a final concentration of 0.5% and 100 g ml^{-1}, respectively. The mixture was incubated for 1 h at 37°C. Precipitation was then performed by adding 0.8 M NaCl and 0.5% hexadecyltrimethyl ammonium bromide solution and incubating for 20 min at 65°C. The solution was extracted with an equal volume of Roti® phenol-$CHCl_3$ mixture. DNA was precipitated with 0.6 volume of isopropanol. The resulting DNA pellet was washed with 70% ethanol, centrifuged at 8,000 rpm for 30 min at 4°C and air dried. DNA pellet was then dissolved in TE buffer. Genomic DNA was analyzed and visualized by agarose gel according to the method of Sambrook et al. (1989). The gel was prepared with 0.8% (w/v) agarose dissolved in TBA buffer (0.89 M Tris, 0.02 M EDTA-Na_2-salt, 0.89 M Boric acid, pH 8.5). The run was performed at 77 V. Gels were stained with Sybr Green. Genomic DNA was restricted with *Eco*R1 according to the instructions described by Sambrook et al. (1989).

Table 1. Physiological effect of *E.coli* and *B. thuringiensis* on the total haemocyte counts.

Time post infection (h)	Average no.	Haemocytes/mm^3			
	E. coli	*E. coli*	*B. t$_1$*	*B. t$_2$*	*B. t$_3$*
24	38500±16^a	38500±16^a	18000±15.7^c	17333.3±14.5^c	14000±25^d
36	43667±19.11^a	43667±19.11^a	20333.3±23^c	19334± 31^c	15000±11.5^d
48	69500±20.2^a	69500±20.2^a	31176.9±37^b	24000±36^c	17666±20^d

- Each value presents the mean ± SE. Statistical analysis between control and treatment; there are no significant differences among means with same letter. The different letter(s) are significant at $p \leq 0.05$ according to the LSD test.

Statistical methods

Data were statistically analyzed to check the significance of differences between treatments and control using F test and LSD (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Haemocyte counts

The total number of circulating haemocytes in an insect varies with developmental and physiological stages (Essawy, 1990). Four primary types of haemocytes were observed in the haemolymph of the *B. mori* fifth larval instar. There were prohaemocyte (Pr), plasmatocyte (Pl), granulocyte (Gr) and oenocytoide (Oe). Granulocytes were the most abundant haemocytes followed by Pl. Oecytoides were numerically less abundant.

E. coli

A marked increase in THC was noted at 48 h post-treatment as compared to control with 69500 and 57690 cells/mm^3, respectively (Table 1). Also, the present results revealed a slight increase in the THC at 24 h of *E. coli* treatment but still increased as compared to control values, 38500 cells/mm^3. These results revealed that *E. coli* treatment increased the THC of *B. mori* by 59.09, 33.1 and 20.47% as compared to THC of control after 24, 36 and 48 h, respectively. The results obtained are in contrast to those detected by Ericsson et al. (2009) who reported that there was significant reduction in the haemocyte count after the injection of *Trichoplusia ni* with *E. coli* and Dunphy and Nolan (1982) who found that THC was initially declined when the larvae were injected with protoplasts, growth medium (MGM) or *E. coli*.

The percentage of the number of Pr increased at 24, 36 and 48 h post-infection, 5.8, 7.6 and 6.6%, respectively. As shown in Figure 1a, b, c and d the infection of *B. mori* fifth larval instar with *E.coli* markedly increased the percentage of the number of Pl and Gr at 48 h post-infection to about 16.7 and 35%, respectively.

These results may be due to the important role of plasmatocytes and granulocytes in the insect immunity against bacterial infection. The data also revealed that Pr, Gr and Pl increased at 24, 36 and 48 h post-infection, also Oe increased at the same time to 15.38, 17.6 and 16.6%, respectively. These results are in accordance with the findings of Horohov and Dunn (1983) who reported that bacterial injection into *Manduca sexta* larvae caused significant changes in the number of oenocytoides.

B. thuringiensis

The dynamic reactions of the 5th larval instar of *B. mori* against *B. thuringiensis* revealed marked decrease in the total haemocyte count (THC).

Quantitative analysis of THC of insects infected with *B. thuringiensi*s gave the results shown in Table 1. Bacterial injection at a concentration of 1.5 ppm led to significant decrease in THCs after 24, 36 and 48 h post-injection reaching to 14000, 15000 and 17666 cells/mm^3, respectively, while in the control THCs were 24200, 32800 and 57690 cells/mm^3, respectively. Meanwhile, the injection of *B. mori* with *B. thuringiensi*s at concentration of 0.5, 1 and 1.5 ppm decreased the THCs to about 45.9, 58.39 and 69.4% after 48 h post-infection, respectively, as compared to the control.

Similar results were reported by Ericsson et al. (2009) who studied the immune response to *B. thuringiensi*s Kursraki. (Btk) in susceptible (Bt–Rs) and resistant (Bt-R) *Trichoplusia ni* after exposure to low doses of Btk. They reported a reduction in haemocyte counts after exposure to Btk. Also, Johnson (1981) investigated that haemocytes were lost from the circulation by their incorporation into aggregations or by lyses of individual cells after injection of *Homarus amrericanus* with bacteria. Also, the present data are in agreement with those obtained for *Agrotis ipsilon* larvae infected with *B. thuringiensi*s (Abd El-Aziz and Awad, 2010). On the other hand, the obtained results were in contrast to those detected by Horohov and Dunn (1983) who found a marked increase in THC of *M. sexta* larvae injected with *Pseudomonas aeruginosa*.

As shown in Figure 1 a, b, c and d, it is clear that the injection with *B. thuringiensis* at all concentrations decreased the percentage of the number of Pr, Gr and Pl counts especially at 48 h post-infection. The maximum decrease was observed at concentration of 1.5 ppm. The percentage of the number of Pr decreased at 24, 36 and 48 h post-infection to about 20, 37.3 and 42%, respectively. Furthermore, the injection of the 5th larval instar of

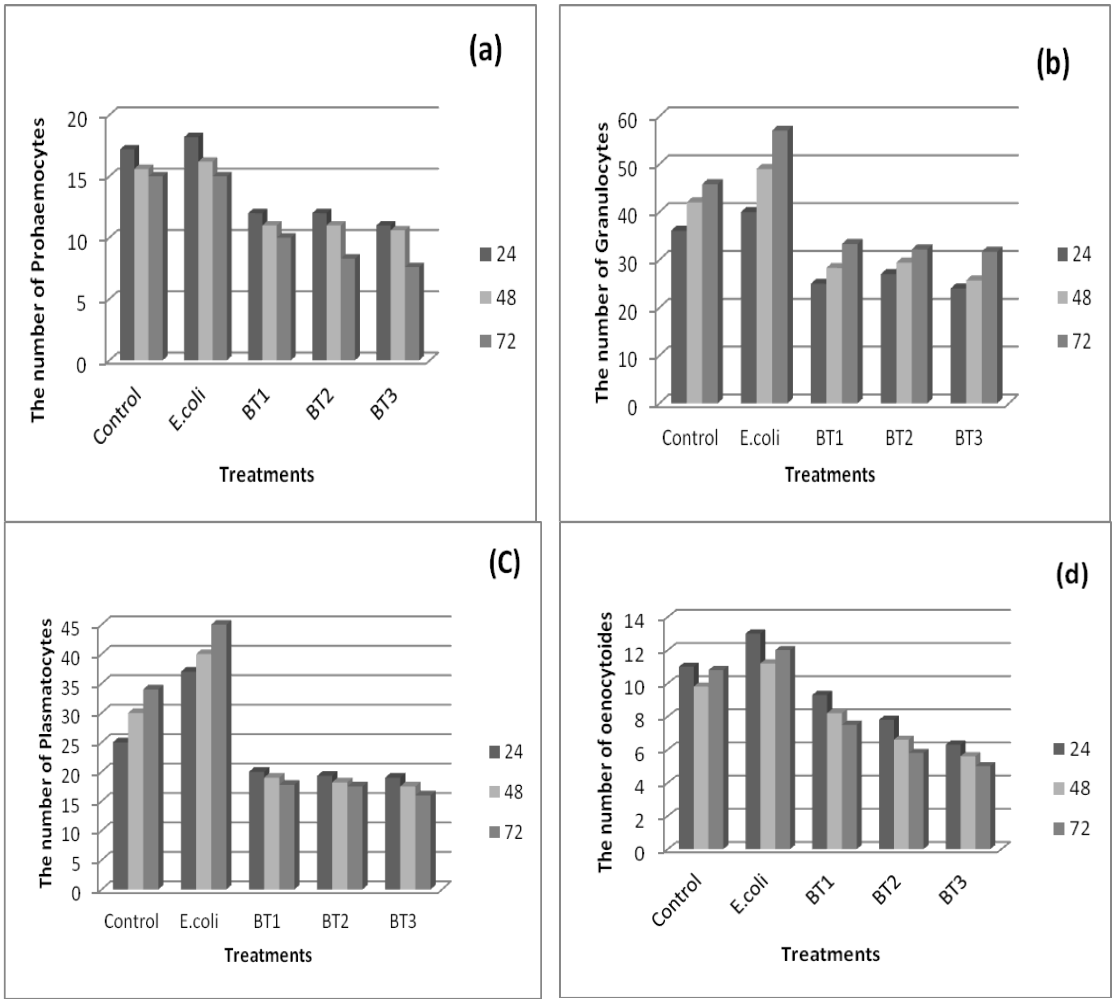

Figure 1. Effect of *E.coli* and *B. thuringiensis* on the different haemocyte count of the *B. mori* 5th larval instar. Where; (BT1 & BT2& BT3): *B. thuringiensis* at concentration 0.5 , 1 and 1.5 p.p.m, respectively. a) Prohaemocyte; b) Granulocyte; c) Plasmatocyte; d) Oenocytiode.

B. mori with *B. thuringiensis* at the same concentration decreased the Gr, Pl counts to about 27.4 and 37% as compared to the control, respectively, at 48 h post-infection .The same trend was observed in the Oe counts which decreased to about 52.6%, less than control.

These results are in accordance with the findings of Horohov and Dunn (1983) who reported that the bacterial injection into *Manduca sexta* larvae caused a significant decrease in the percentage of Grs and Pls. Also, Chain and Anderson (1982) reported a direct demonstration that *B. cereus* injection can cause a selective removal of plasmatocytes from the circulating haemolymph of *Galleria mellonella.* Perhaps the injection with bacteria in some way causes Pls to clump together or to cling to the lining of the haemocel (Abd El-Aziz and Awad, 2010).

The hormonal regulation of immunity during the last larval instar of *B. mori*

Results reported in Figure 2 depicted that the infection of *B. mori* fifth larval instar with *E. coli* markedly increased the CA surface area at 72 and 120 h post-infection to about 18.9 and 24.9%, respectively of the control. This increase may be due to the effect of *E. coli* on the immunity response of larvae .These results are in accordance with findings of Freitak et al. (2007) who demonstrated that injection of Gram negative bacteria *E. coli* can induce the immune response of lepidopteran larvae.

In contrast, injection of *B. mori* fifth larval instar with *B. thuringiensis* caused a sharp decrease in the CA surface area up to 96 h post-infection to reach the minimum value of about 54.5% less than the control which is followed by a marked increase of CA surface area at 120 h to about 12.18% over the contol. Perhaps, the infection with bacteria in some cases caused inhibition of CA activity during few days of infection and the increase of the CA activity after that may be due to the release of antibacterial peptides as an immunity response against bacteria which helped the gland to reprogramme itself in its cycle during the last larval instar. The presented results are consistent

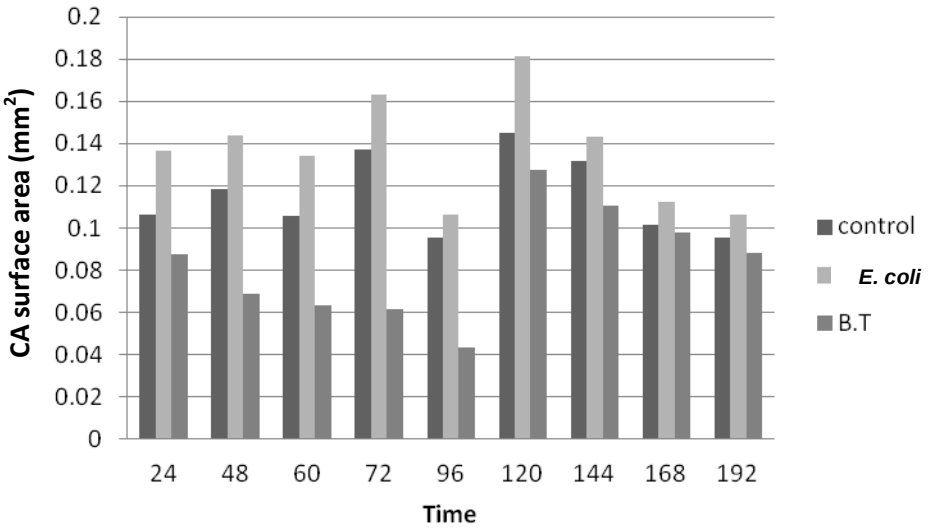

Figure 2. Physiological effect of *E. coli* and *B. thuringiensis* on the corpora allata surface area.

with those detected by Tian et al. (2010) who suggested that JH plays a positive role in the regulation of innate immunity in the larval fat body. Also, Riddiford (2003) suggested that JH has a significant role in the control of immune humoral function. A number of studies in *Drosophila* imply that 20E induces AMP mRNA expression and acts as an immune-activator (Meister et al., 1996; Silverman et al., 2000) while JH acts as an immune-suppressor by antagonizing 20E signaling (Flatt et al., 2008). Also, Flatt et al. (2005) found that juvenile hormone regulate the immunity of *Drosophila* by inhibiting phenoloxidase (Po) synthesis and prevents cuticular melanization.

In the light of the foregoing results, it could be concluded that the fluctuations that occurred in THC and CA surface area (as an indicator of its activity) are going in the same direction. These results are similar to those obtained in previous studies of Gad (1996) who noted a positive correlation between the CA volume and THC.

It is important to go deep to explain the side effects and the mode of action by which the injection of the bacteria can effect the physiology and genetics of *B. mori*. A molecular marker of *B. mori* DNA is the most important method for determining the affected region on DNA (Williams et al., 1990) since they reveal DNA polymorphisms among genetically related individuals. A similar strategy has been used to identify the nucleopolyhedrovirus (NPV), another important silkworm genotype virus (Yao et al., 2003). *B. thuringiensis* is the most widely used microbial pesticides. The biochemical basis of the pesticide is an insecticidal crystal protein (ICP), which is produced by the bacterium as a 133-kDa protoxin that requires proteolytic cleavage in the insect gut for activation. The mutagenic effect of the bacterial injection produced several proteins that enable it to kill insects through the alteration of the physiological processes (Brown et al., 2004).

B. thuringiensis Cry1Aa insecticidal protein is the most active known *B. thuringiensis* toxin against the forest insect pest Lymantria dispar (gypsy moth), unfortunately it is also highly toxic against the non-target insect *B. mori* (silk worm). In fact, it was found in previous studies that *B. thuringiensis* produces different types of insecticidal crystal proteins (ICPs) or delta-endotoxins. The type A protein from *B. thuringiensis* var. kurstaki HD-1 was found to be 400 times more active against *B. mori* than type C protein from *B. thuringiensis* var. kurstaki HD-244) (Brown et al., 2004). To locate the specificity domain of the type A protein for *B. mori*, site-directed mutagenesis was used to introduce or remove restriction enzyme sites, facilitating the exchange of regions of the two genes. The hybrid genes were overexpressed, and purified ICP was used in bioassays. *The B. mori* specificity domain for the ICP A toxin is located in the amino-terminal portion of the hypervariable region between amino acids 332 and 450.

It may be assumed that differences in restriction sites are due to amino acid differences in proteins of *B. thuringeinsis* and *E. coli* against *B. mori*. The change of a portion in specific regions of *B. mori* DNA is an indicator of mutagenic effects occurring in the protein.

Our results confirm that this the genotoxicity occurred in *B. mori*, in a DNA band region leading us to predict that this region is composed of several structural domains that are disrupted by the toxin secreted by both *B. thuringeinsis* and *E. coli* against *B. mori* (Figure 3).

Gillespie et al. (1997) and Dettloff et al. (2001) reported that haemocyte responses were triggered by antigens adhering to two types of receptor: pattern recognition and promiscuous non-pattern recognition receptors. The receptors must transfer information through signal transduction pathways into the haemocytes to continue the anti-antigen responses; that is, the release of opsonins and/or adhesive proteins. Haemocytes of the silkworm *B.*

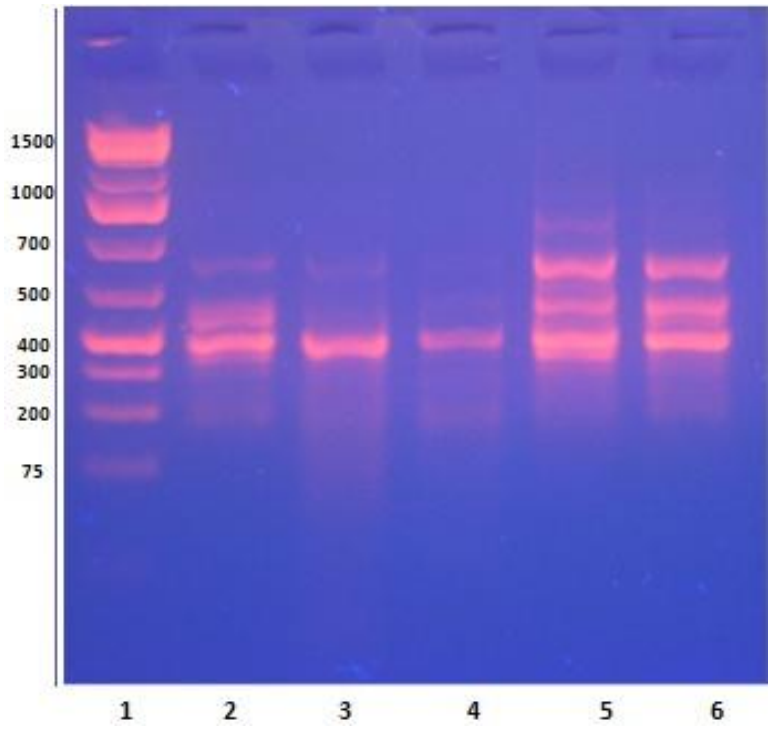

Figure 3. Detection and localization of the affected *B. mori* larval DNA treated with *E. coli* and *B. thuringiensis*. Lane 1: Marker 1500 bp; 2: Control
3: Treatment with *E. coli*; 4: Treatment with *B. thuringiensis* (0.5 ppm); 5: Treatment with *B. thuringiensis* (1 ppm); 6: Treatment with *B. thuringiensis* (1.5 ppm).

mori require an unidentified PKA isotype to produce cecropins (antibacterial proteins) (Choi et al., 1995; Shimabukuro et al., 1996). Based on the use of H-89, an inhibitor of PKA isotypes, a type of PKA may limit using several experimental approaches (luminometry, spectrophotometry, fluorimetry); we found no increase in ROS production in the hemolymph of *B. mori* (Hyrsl et al., 2004). Research conducted by Anderson et al. (1973) showed that the hemocytes of *Blaberus craniifer* (Blattodea) did not reduce NBT in response to zymosan. Whitten and Ratcliffe (1999) provided evidence for the existence of an immune response resembling the respiratory burst in the hemolymph and hemocytes of the cockroach, *Blaberus discoidalis* (Blattodea). The granulocytes of *Bombyx*, which play a key role in phagocytosis in normal larvae was reported by Akai and Sato (1973).

Conclusion

In conclusion, *E. coli* injection has lead to a significant increase in THCs as compared to the control. The injection effect of *E. coli* improved the immune system of the larvae as a result of the larval immune response when compared with the injection effect of *B. thuringiensis*. This was observed in the increase in the total number of haemocytes, the activity of Corpora allata gland and Juvenile hormone secretion as an immune stimulant.

The results demonstrated that *B. thuringiensis* induced the host strong response. Huang et al. (2009) suggested that injection of *B. mori* with *Bacillus bombysepticus* (*Bb*) caused a lot of basal metabolic pathways which were significantly modulated. Furthermore, genes of juvenile hormone synthesis and related metabolism showed up regulation, suggesting that juvenile hormone participate in host modulation during the infection. Moreover, host cellular and systemic immune responses are also induced. Similar to *B. thuringiensis* (Bt), Bb can also induce a silkworm poisoning-related response.

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African Journal of Microbiology Research

Full Length Research Paper

Purification and characterization of extracellular acidophilic α-amylase from *Bacillus cereus* MTCC 10205 isolated from soil

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Amylase from *Bacillus cereus* MTCC 10205 was purified 20.41 with 11.82% recovery by ammonium sulfate precipitation, gel filtration chromatography through Sephadex G-100 and ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose. The final enzyme preparation was pure to near homogeneity as judged by native-polyacrylamide gel electrophoresis (PAGE). The enzyme had a molecular weight of 55 kDa as determined by gel filtration and a single band of 55 kDa as determined by sodium dodecyl sulfate-polycrylamide gel electrophoresis showing it to be a monomer. The purified enzyme had temperature optima of 55°C and pH optima of 5.5. The enzyme retained 72% of its original activity after 90 min of incubation and exhibited gradual loss in activity when incubated at higher temperature. At 60°C after 90 min of incubation, the enzyme was completely inactive. The enzyme appeared to be quite stable at 4°C as it could be stored upto five days with 10% loss in activity, whereas at 35°C, the enzyme lost 28% of its activity just after three days of storage. Inhibition studies revealed SH groups to be involved at the active site of the enzyme.

Key words: Amylase, *Bacillus cereus,* gel-filtration, purification, sodium dodecyl sulphate.

INTRODUCTION

Amylases are of ubiquitous occurrence and are holding maximum market share of enzyme sales (Sivaramakrishanan et al., 2006). These hydrolyze starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units (Windish and Mhatre, 1965) and hence are used in a wide range of starch industries that is brewing, baking, starch liquefaction and distillery (Souza and Magalhães, 2010). Amylases that are active at acidic pH are generally used in the glucose syrup industry, whereas those active at basic pH are explored in detergent industries (Tonokova, 2006). Although amylases can be derived from several sources such as plants, animals and microorganisms, microbial sources are the most preferred one for large scale production in meeting industrial demands (Rao et al., 2007). The microbial amylases are usually extracellular and are widely distributed in bacteria, actinomycetes and fungi (Sivaramakrishanan et al., 2006). Two major classes of amylases have been identified in microorganisms, namely amylase

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Abbreviations: SM, Starch medium; **DNSA,** 3, 5- dinitrosalicylic acid; **DEAE,** diethyl aminoethyl; **PAGE,** polyacrylamide gel electrophoresis; **PHMB,** para hydroxyl mercuric-benzoate; **PMSF,** phenyl methoxy sulfonyl fluoride; **DTNB,** 5, 5-dithiobis-2-nitrobenzoic acid; **EDTA,** ethylenediaminetetraacetic acid; **β-ME,** β-mercaptoethanol.

α-amylase and glucoamylase. The α-amylases (endo-1,4-α-D glucan glucohydrolase, EC.3.2.1.3) are extra cellular enzymes that randomly cleave 1,4-α-D-glucosidic linkages between adjacent glucose units in linear amylose chain. Glucoamylase (exo-1,4-α-D glucan glucohydrolase, EC.3.2.1.1) hydrolyses single glucose unit from the non-reducing end of amylose and amylopectin in a stepwise manner. We report here purification and characterization of acidic amylase from a new strain of bacteria having potential of being industrially used.

MATERIALS AND METHODS

Reagents

All the chemicals used in the present work were of analytical grade and were purchased from Sigma Chemicals Co., USA, Hi-Media, Sisco Research Laboratories and E. Merck, Bombay.

Bacterial strain and growth conditions

Microorganisms isolated from the soil collected from vegetable and grain market, were screened for amylase production. The most efficient producer was identified as *Bacillus cereus* MTCC-10205 (Institute of Microbial Technology-IMTECH Chandigarh India). This strain was grown at 35°C from an inoculum containing 2.55 x 10^6 cell mL^{-1} in starch medium (SM) for 36 h under submerged conditions. Inoculum was in a proportion of 2%. Starch medium contained soluble starch 10.0 g L^{-1}, yeast extract 5.0 g L^{-1}, peptone 3.0 g L^{-1}, $MgSO_4$ $7H_20$ 0.2 g L^{-1}, NaCl 0.1 g L^{-1} and K_2HPO_4 0.8 g L^{-1} (pH 7.0).

Amylase assay

Amylase activity was determined through spectrophotometrical measure of dinitrosalicylic acid reduction by reducing sugars released from soluble starch used as substrate (Miller, 1959). The reaction mixture contained 0.80 mL starch (10 g L^{-1} in 0.016 M sodium acetate buffer, pH 4.8) and 200 μL of enzyme solution in a final volume of 1 mL. The reaction was incubated at 40°C for 30 min and stopped by adding 2 ml of 3, 5- dinitrosalicylic acid (DNSA) reagent (1% DNSA, 0.05% sodium sulphide, 30% sodium potassium tartarate and 0.2% phenol in 0.4 N NaOH). The mixture was heated for 5 min in boiling water bath and then cooled to room temperature. Absorbance of sample was measured at 540 nm against the substrate blank. A standard curve of maltose ranging from 0 to 1000 µg/ml was constructed and then the released maltose was determined in the samples from standard curve. One unit of amylase activity was defined as the amount of enzyme that liberated 1 nmol of maltose equivalent under the experimental conditions in 1 min. Amount of soluble protein was determined according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

Enzyme purification

B. cereus MTCC-10205 grown in SM for 36 h was filtered through muslin cloth. The filtrate containing amylase was centrifuged at 12,000 rpm for 15 min to obtain cell-free supernatant which was referred to as crude extract. The crude extract was subjected to 45-70% $(NH_4)_2SO_4$ saturation, centrifuged at 12,000 rpm for 15 min and precipitates dissolved in 0.016 M sodium acetate buffer (pH 4.8) and dialyzed against same buffer. The enzyme preparation obtained after ammonium sulphate fractionation was carefully layered over the top of a Sephadex G-100 column (700 x 15 mm) and eluted with 0.016 M sodium acetate buffer pH 4.8, at a flow rate of 10 ml/h. Fractions of 3 ml each were collected and protein content (A_{280}) and enzyme activity analyzed. The fractions with amylase activity were pooled and concentrated by dialyzing against sucrose. The concentrated enzyme was loaded on a diethyl aminoethyl (DEAE)-cellulose column (300 x 30 mm). The column was washed with 0.016 M sodium acetate buffer (pH 4.8) and amylase was eluted by linear gradient of NaCl (0- 0.4 M) in the same buffer. Purity of enzyme was checked by native-polyacrylamide gel electrophoresis (PAGE).

Purity of enzyme by native-PAGE

Purity of the final enzyme preparation obtained after DEAE-cellulose chromatography was checked by native-PAGE (10% gel) on slab gels (M/S Atto, Japan) using anionic system (Davis, 1964).

Sample preparation

200 µl of each enzyme preparation *viz*., crude $(NH_4)_2SO_4$ fraction, Sephadex G-100 fraction and DEAE-cellulose fraction was taken in an eppendorf tube. To it, 0.2 ml glycerol (20%) and 25 µl of 5% bromophenol blue were added and contents mixed thoroughly.

Electrophoresis

The clean plates were sealed by tygon tubing, clamped to make a mould and 10% resolving degassed gel solution was poured. A layer of water was then gently overlaid using a syringe. The assembly was left undisturbed for polymerization of the gel which took about 30 min and was indicated by a sharp interface between water and gel. Water was removed, 3.75% stacking gel solution poured and comb inserted immediately with care so that no air bubble was trapped beneath it. After polymerization, comb and tygon tubing were removed. Gel plates were fixed to the electorphoretic apparatus. Sample wells were rinsed with electrode reservoir buffer and the two reservoirs were filled with electrode buffer. Samples containing 200 µg protein were loaded in separate wells and electrohoresis was carried out at a constant current of 10 mA for first 30 min followed by 20 mA constant current till the tracking dye (bromo phenol blue) reached one cm away from the lower end of the gel.

Gel staining

Gel was removed from glass plates and stained overnight with staining solution (2.5% Coomassie Brilliant Blue containing methanol and acetic acid in the ratio of 40:7). The excess stain was removed by diffusion in destaining solution (7.5 % acetic acid and 5.0% methanol). After complete destaining, gel was transferred to 7% acetic acid and photographed.

Molecular weight determination

The molecular weight of purified amylase was determined by molecular exclusion chromatography through Sephadex G-100

Table 1. Summary of purification of amylase from *Bacillus cereus* MTCC 10205.

Purification step	Volume (ml)	Total activity* (U)	Total protein (mg)	Specific activity (U mg^{-1} protein)	Fold purification	Yield (%)
Crude extract	485	106908.5	820	130.37	-	100
$(NH4)_2SO_4$fraction (45-70%)	15	24923	44.38	561.58	4.30	23.31
Sephadex G-100	36	20269.61	12.62	1606.15	12.32	18.95
DEAE-Cellulose	42	12639.03	4.75	2660.85	20.41	11.82

*One enzyme unit is the amount of enzyme that liberated 1nmol of maltose equivalent under the experimental conditions in 1 min.

column (650 x 15 mm). The void volume was calculated by passing blue dextran (2 mg mL^{-1}) through the column. The column was calibrated with standard molecular weight markers; cytochrome-C (12.4 kDa), carbonic anhydrase (29.0 kDa), bovine serum albumin (66.0 kDa), alcohol dehdyrogenase (150.0 kDa) and β-amylase (200.0 kDa) and then made protein free by running about 5 bed volumes of 0.016 M sodium acetate buffer (pH 4.8) through the column. The purified enzyme preparation was loaded over the top of the column and eluted with 0.016 M sodium acetate buffer (pH 4.8) at a flow rate of 15 mL h^{-1}. The fractions of 3.0 ml each were collected and analyzed for protein (A280) and amylase activity. The active fractions were pooled and their volume determined. A plot of log of molecular weight versus elution volume gave the molecular weight of the enzyme.

Polypeptide composition and their molecular weight

SDS-PAGE was performed according to Laemmli (1970) to determine the polypeptide composition and their molecular weight. The composition of the gel was same as in case of native-PAGE except that it contained 2% SDS. The samples and the standard proteins were mixed with equal volume of 2X buffer (0.250 M Tris-HCl pH 6.8 containing 4% SDS and 10% β- mercaptoethanol, 20 % glycerol and 0.4% bromophenol blue) and boiled for 5 min. The standard molecular weight protein markers used were galactosidase (175.0 kDa), paramyosin (83.0 kDa) MBP-CBD (62.0 kDa), aldolase (47.5 kDa), triosephosphate isomerase (32.5 kDa), β-lactoglobulin B (24.0 kDa), lysozyme (16.5 kDa) and aprotinin (6.5 kDa). Molecular weight of the protein was determined from the standard curve of Rm values against log molecular weight of the standard molecular marker proteins.

$$Rm = \frac{\text{Distance travelled by protein band}}{\text{Distance travelled by tracking dye}}$$

Characterization of purified enzyme

pH, temperature optima and Km value

Optimum pH of the enzyme was determined by carrying out amylase assay at different pH values ranging from 3.5 and 8.5 at 40°C. The buffers used were acetate buffer for pH 3.5 to 5.5, phosphate for 6.0 to 7.5 and Tris-HCl for pH 8.0-8.5. Optimum temperature was determined by amylase activity at different incubation temperatures ranging from 30 to 70°C at pH 5.5. To study the effect of substrate (starch) concentration, enzyme activity was determined over a wide range of starch concentration ranging from 1.0 to 14 g L^{-1}. The Km was determined from reciprocal Lineweaver and Burk (1934) plot.

Thermostability, pH stability and storage ability

To determine enzyme thermostability, the amylase extract was incubated at 40, 50 and 60°C and residual enzyme activity was measured at 15 min interval up to 60 min at optimum conditions of pH and temperature (pH 5.5, 55°C). The amylase stability against pH was analyzed by its residual activity under optimum conditions (pH 5.5, 55°C) after incubating the enzyme in buffers with pH value ranging from 3.5 and 8.5 at 30°C for 1 h. The enzyme stability at storage conditions of 4 and 35°C was assessed by measuring residual activity weekly for 30 days.

Effect of metal ions and other additives

To determine the effect of several metal ions and other additives, the enzyme solution was incubated with 1 and 5 mM solution each of $MgSO_4$, KCl, $MnCl_2$, $MnSO_4$, $ZnSO_4$, $FeCl_3$, $CuCl_2$, $CaCl_2$, SDS, β-ME, iodoacetate, para hydroxyl mercuric-benzoate (PHMB), phenyl methoxy sulfonyl fluoride (PMSF) and 5, 5-dithiobis-2-nitrobenzoic acid (DTNB) at 30°C for 30 min. The enzyme activity was determined as described earlier at optimum conditions (pH 5.5, 55°C).

RESULTS AND DISCUSSION

Purification of amylase

Amylase from *Bacillus cereus* MTCC 10205 was purified using conventional techniques of enzyme purification such as ammonium sulphate precipitation, gel filtration through Sephadex G-100 and ion exchange through DEAE-cellulose. Summary of enzyme purification is given in Table 1. The ammonium sulphate precipitation accounted a purification factor of 4.30 fold and 23% recovery. Concentrated enzyme obtained after $(NH_4)_2SO_4$ fraction was loaded on pre-equilibrated Sephadex G-100 column. The elution profile of the proteins and enzyme activity (Figure 1A) showed a single narrow peak of amylase activity comprising fractions 38-49, which coincided with one peak of protein. This step increased the purification factor to 12.32 fold recovering an 18.95% of the total activity as compared to the crude extract.

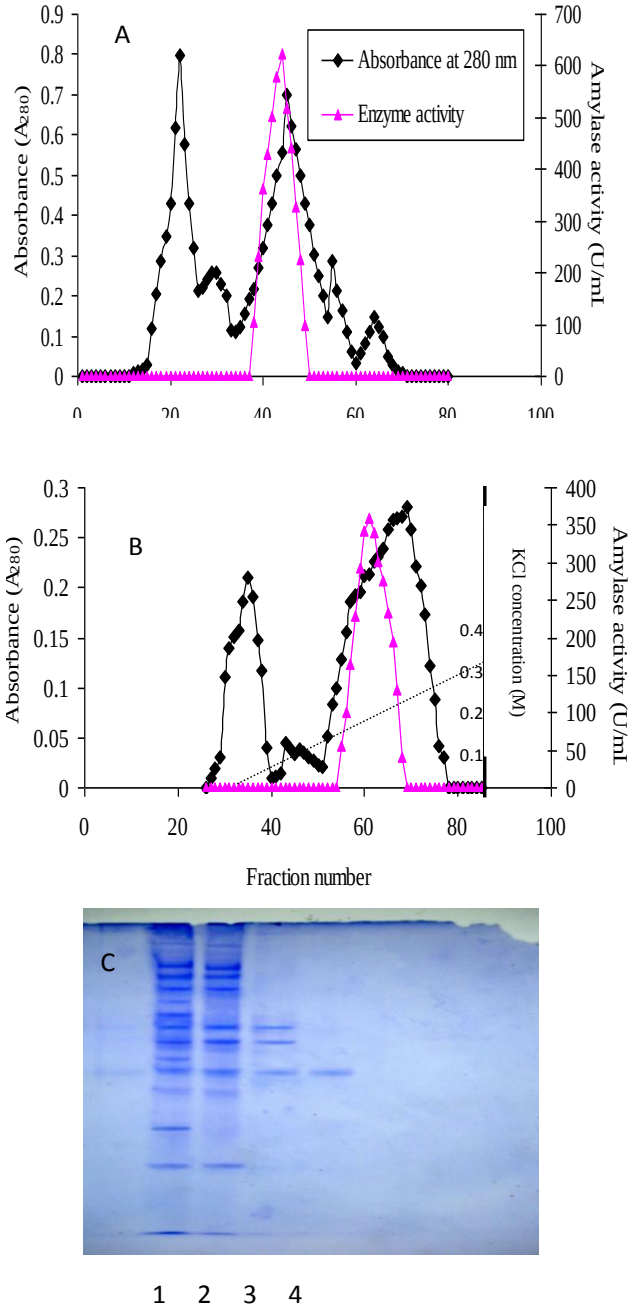

Figure 1. Elution profile of amylase from *Bacillus cereus* MTCC 10205 on Sephadex G-100 [A] and DEAE cellulose [B]. The enzyme activity was shown as -▲- and absorbance at 280 nm (amount of protein) was shown as -♦- which indicated the amount of protein. Lane 1, Electrophoretic pattern of amylase fractions during purification on native-PAGE [C] crude extract; Lane 2, ammonium sulphate fraction; Lane 3, Sephadex G-100 fraction; Lane 4, DEAE-cellulose fraction.

Elution of ion exchange chromatography on DEAE-cellulose also showed a single peak of amylase activity coinciding with one main protein peak between fractions 55-68 (Figure 1B). This purification process resulted in an enzyme preparation 20.40 fold purified with specific activity of 2660.9 U/ mg protein and a

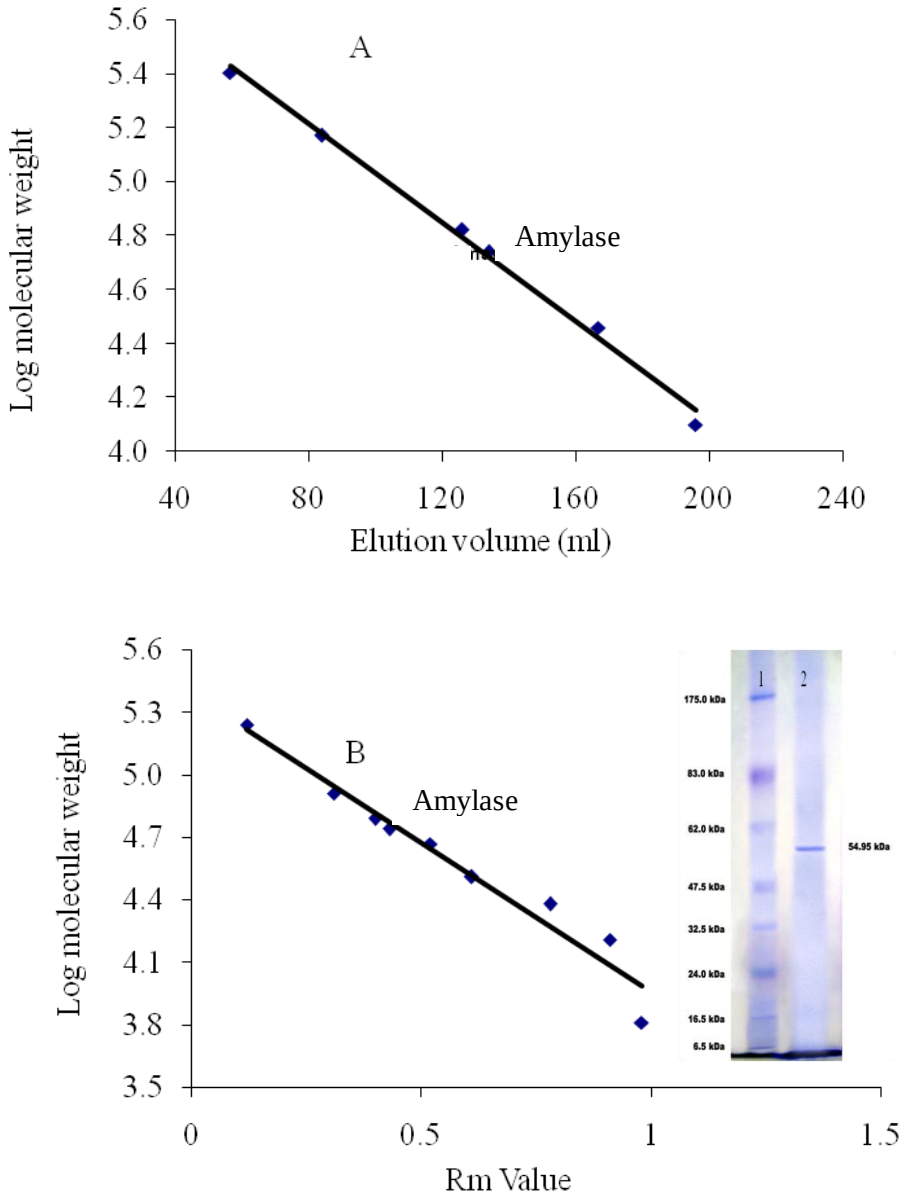

Figure 2. Determination of molecular weight and sub unit composition of purified amylase from *Bacillus cereus* MTCC 10205 using gel filtration through Sephadex G-100 [A] and SDS-PAGE [B]. Lane 1, molecular mass markers: β-galactosidase (175kDa), paramyosin (83.0 kDa), MBP-CBD (62 kDa), aldolase (47.5 kDa), triosephosphate isomerase (32.5 kDa), β-lactoglobulin B (24 kDa), lysozyme (16.5 kDa) and aprotinin (6.5 kDa). Lane 2, amylase purified from *B.cereus.*

yield of 11.82% (Table 1). The purity of the enzyme at each step of purification was analyzed by native polyacrylamide slab gel electrophoresis (Figure 1C). Sixteen major bands were detected in crude preparation whereas only 11 bands were observed after ammonium sulphate fractionation. Sephadex G-100 fraction gave 3 bands while final purified enzyme obtained after DEAE-cellulose column gave one major band suggesting that the enzyme was purified to near homogeneity.

Amylases have been purified from various microorganisms by similar purification processes to that followed in this work. The purification degree of amylase achieved in the present study was similar to that reported by Bano et al. (2009) from *Bacillus subtilis* KIBGE-HAS in a two step process including ammonium sulfate precipitation and ultrafiltration (purification factor: 19.2 fold;

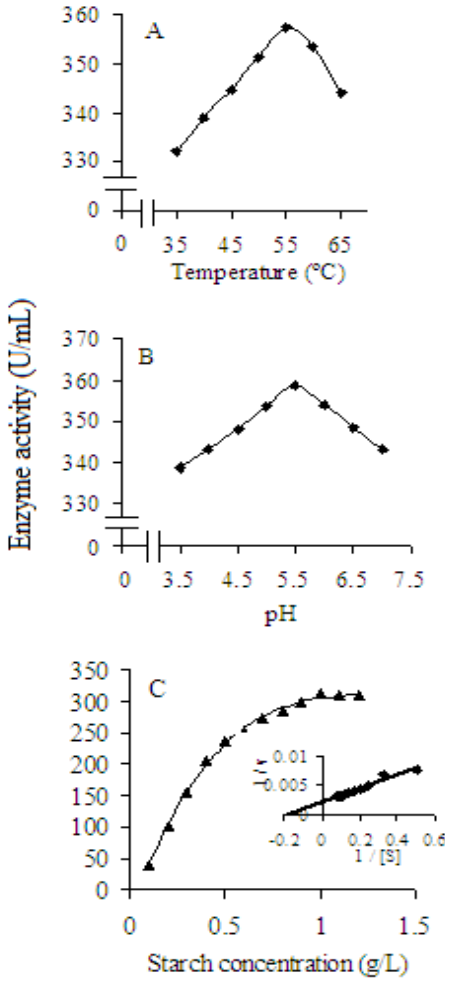

Figure 3. Characterization of purified amylase from B. cereus MTCC 10205 for optimum temperature [A], optimum pH [B] and Km value [C].

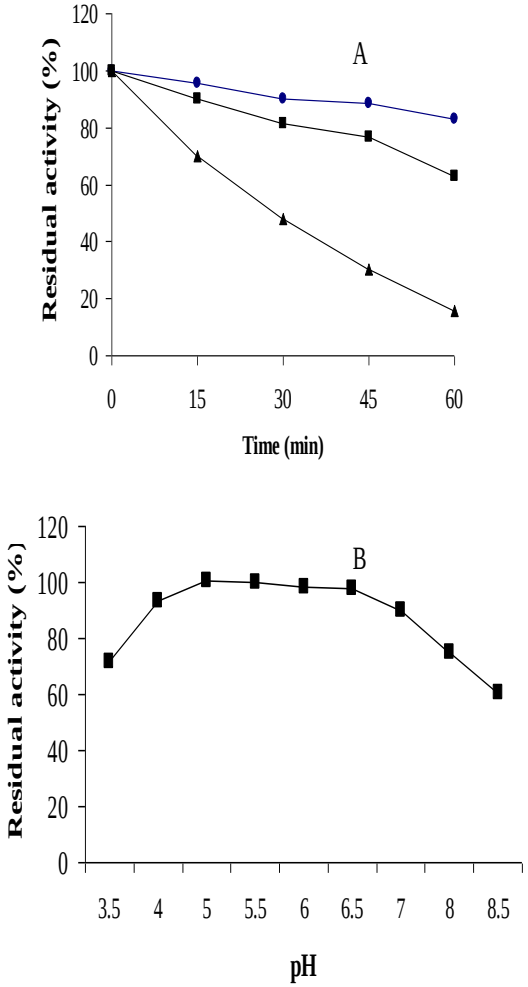

Figure 4. Thermostability (A) and pH stability (B) of the amylase purified from Bacillus cereus MTCC 10205. The enzyme was incubated at 40°C (–●–), 50°C (–■–) and 60°C (–▲–) for its thermostability.

specific activity: 4195 U/ mg protein). Similarly amylase from *B. subtilis* US 116 was purified to near homogeneity by using a combination of acetone preci-pitation, size exclusion and ion-exchange chromatogram-phy (Messaoud et al., 2004). However, the amylase from *B. licheniformis* was purified 20..3 fold with 23.62% yield by ion-exchange chromatography on DEAE-cellulose and gel filtration on BioGel P100 column (Adeyanju et al., 2007).

Molecular weight

From gel filtration results, the molecular weight of native amylase was estimated to be 55.0 kDa (Figure 2A). Electrophoresis of purified amylase in SDS-PAGE/showed a single band with similar molecular weight (54.95 kDa). These results indicate that amylase from *B. cereus* is a monomer (Figure 2B). Amylases of almost similar molecular weight have been reported from *B. cereus* NY-14 (Yoshigi et al., 1985), *Bacillus subtilis* (Uyar et al., 2003), and *Bacillus* sp. AB 68 (Aygan et al., 2008). Endoamylases are almost exclusively single subunit proteins, however, some amylases especially those having large molecular weight are found to possess more than one subunit as reported in *Pyrococcus furiosus* (Laderman et al., 1993) and *Bacillus* sp. A3-15 (Arikan , 2008).

Temperature and pH optima and Km value

The maximum activity of purified amylase from *B. cereus* (357.48 U/ mL) reached at 55°C (Figure 3A). Higher incubation temperatures of reaction led to a gradual loss

of activity. The temperature optima observed during the present investigations is in accordance with the temperature optima reported in *B. cereus* (Yoshigi et al., 1985). Thermostable amylases having temperature optima between 50 to 60°C have also been isolated from a number of sources including *Bifidobacterium adolescentis* (Lee et al., 1997), *Aspergillus oryzea* (Ramachandran et al., 2004), *B. subtilis* (Bezerra et al., 2006) and *Bacillus* sp. AB68 (Aygan et al., 2008). The themostable alpha amylases isolated from various species of Bacillus have been preferred for use in starch processing industry (Nigam and Singh, 1995)

Figure 3B depicts that enzyme had maximum activity at pH 5.5 (358.84 U/ mL). At pH above and below 5.5, the activity decreased. The optimum pH for amylase from *B. cereus* MTCC-10205 was different from that of *Alicyclobacillus acidocaldarius* which have been reported to have acidic pH optima of 3.0 (Schwermann et al., 1994) and from that of the alkaline amylases from *B. cereus* (Annamalai et al., 2011), *Bacillus cohnii* US 147 (Ghorbel et al., 2009) and *Bacillus* KSM K-38 (Hagihara et al., 2001) with pH optima of 8, 9 and 10, respectively. However, amylases with stability in a narrow range have also been reported in *Halomonas meridiana* (Coronado et al. 2000). The amylases working in the pH range of 5.0-6.0 are preferred for starch industry as this pH range could eliminate unwanted side reactions during starch processing (Vieille and Zeikus, 2001).

With increasing concentration of substrate (1-14 g L^{-1}), the enzyme showed a typical hyperbolic velocity saturation curve (Figure 3C) revealing that it followed Michaelis-Menten kinetics. The enzyme activity increased with increase in starch concentration attaining maximum value at 10 g L^{-1} starch, above which the enzyme activity remained almost constant suggesting that the enzyme got fully saturated at this concentration. From the double reciprocal Lineweaver Burk plot, the Km of the enzyme was found to be 5.37 g L^{-1}. The Km value observed during the present study reveals higher affinity for the substrate than that observed for amylase from *B. cohnii* US 147 whose Km value is reported to be 7.0 mg/mL (Ghorbel et al., 2009). Adeyanju et al. (2007) however, reported that amylase from *Bacillus licheniformis* had sigmoidal kinetics with a Km for soluble starch of 1.097% starch.

Thermostability, storage stability and pH stability

The enzyme showed progressive loss in activity with temperature and with time of incubation (Figure 4A). Amylase retained 83% of its original activity at 40°C after 60 min. At 50 and 60°C after 15 min of incubation, enzyme retained 90 and 70.1% of its original activity whereas after 60 min of incubation, the enzyme showed only 62.90 and 15.6% activity, respectively. The enzyme was stable over a broader pH range retaining >90% of its initial activity after incubation at 30°C in buffers of pH 4.0-7.0 for 1 h (Figure 4A). Thermostable enzymes including amylases, proteases and lipases offer major biotechnological advantages over mesophillic enzyme (McMohan et al., 1999). Bacterial α-amylases possessing high heat resistance have been reported earlier from *Alicyclobacillus* sp.A4 (Bai et al., 2012) *Bacillus* sp. AB-68 (Aygan et al., 2008) and *B. subtilis* KIBGE-HAS (Bano et al., 2009). *Alicyclobacillus* sp.A4 has also been shown to have broader pH stability (Bai et al., 2012).

The purified amylase appeared to be quite stable at 4°C because it could be stored up to 5 days with only 10% loss in activity (Table 2). After that, the activity declined gradually causing 50% loss in activity after 30 days of storage. Storage of the purified amylase at 35°C for 3 days resulted in 28% loss in activity. Further increase in storage period at this temperature led to rapid inactivation of the enzyme showing only 15% of activity after 22 days of storage. However, complete loss in activity was observed after 30 days of storage. In agreement with our results, storage of amylase from *B. subtilis* KIBGE-HAS at 4°C for 124 days retained 70% activity while the storage at 37°C for 25 days resulted in complete loss of activity (Bano et al., 2009).

Effect of metal ions and other additives

Metal ions like K^+ and Zn^{2+} at 1 mM concentration stimulated amylase activity by 31 and 18%, respectively, while at 5 mM concentration, they inhibited activity by 51 and 45% (Table 3). Ca^{2+} however, was stimulatory at both concentrations (1 and 5 mM) whereas other metal ions such as Cu, Mn, Mg, Fe were inhibitory. Ethylene-diaminetetraacetic acid (EDTA) also inhibited amylase activity, suggesting that metal ions were required for the amylase activity.

These results are in accordance with those reported by Kaneko et al. (2005), who observed an increase in amylase activity in the presence of Ca^{2+}. The alpha amylase preparations used for starch liquefaction have been reported to show highest activity at pH 5.5-6.0 and require addition of calcium ion for stability (Vieille and Zeikus, 2001). Similarly, the activity of amylase from *Bacillus* sp. AB-68 has been reported to be inhibited by EDTA by 34% thus indicating the requirement of metal ions (calcium) for its activity (Aygan et al., 2008).

Complete inhibition of enzyme activity in the presence of p-hydoxyl-mercuribenzoic acid (PHMB) and 5,5-dithio-bis(2-nitrobenzoate) (DTNB), and stimulation by β-ME indicated the involvement of SH-group(s) at the active site of the enzyme. Iodoacetate also inhibited the amylase activity further confirming the sulfhydryl residue to be essential for catalytic activity of amylase (Table 3). The present observations are similar to the results obtained from the studies of Ezeji and Bahl (2006), Afifi et al. (2008) and Aygan et al. (2008). However, the enzyme

Table 2. Storage ability of purified amylase from *Bacillus cereus* MTCC 10205.

Storage ability	Enzyme activity* (U/ mL)	
Days of storage	Storage temperature	
	4°C	35°C
0	361.11(100)	361.11(100)
1 (day)	360.31 (99.77)	323.96 (89.71)
2 (days)	357.48(99.01)	282.07 (78.11)
3 (days)	350.35(97.02)	259.98 (72.00)
4 (days)	339.48(94.00)	232.35 (64.34)
5 (days)	325.32(90.11)	205.18 (56.82)
6 (days)	315.70(87.42)	173.13 (47.94)
7 (days)	292.82(81.08)	140.86 (39.00)
8 (days)	279.80(77.48)	112.44 (31.13)
15 (days)	252.40(69.90)	81.20 (22.48)
22 (days)	209.48(58.00)	54.91 (15.20)
30 (days)	182.08(50.42)	-

*****One enzyme unit is the amount of enzyme that liberated 1nmol of maltose equivalent under the experimental conditions in 1 min. ******Values in parentheses indicate % of the control values.

Table 3. Effect of different metal ions and additives on purified amylase from *Bacillus cereus* MTCC 10205.

S/N	Metal ions/additives	Enzyme activity* (U/mL)	
		1 mM	5 mM
1	None	359.52 (100)	359.52 (100)
2	$MgSO_4$	347.63 (96.70)	280.82 (78.10)
3	KCl	470.94 (131.00)	184.23 (51.24)
4	$MnCl_2$	280.71 (78.07)	217.52 (60.50)
5	EDTA	104.17 (28.97)	44.16 (12.28)
6	$MnSO_4$	323.51 (89.98)	161.70 (44.97)
7	$ZnSO_4$	424.18 (117.98)	148.11 (41.19)
8	$FeCl_2$	321.02 (89.29)	297.58 (82.77)
9	$CuCl_2$	287.27 (80.00)	152.75 (42.48)
10	$CaCl_2$	404.47 (112.50)	470.94 (130.99)
11	β-ME	371.75 (103.40)	482.15 (134.10)
12	PMSF	345.48 (96.10)	332.57 (92.50)
13	Iodoacetate	101.00 (28.09)	14.83 (4.12)
14	DTNB	77.34 (21.51)	21.96 (6.11)
15	PHMB	51.06 (14.20)	16.98 (4.72)

*****One enzyme unit is the amount of enzyme that liberated 1nmol of maltose equivalent under the experimental conditions in 1 min. ******Values in parentheses indicate % of the control values.

from *B. adolescentis* (Lee et al., 1997) was reported to have groups other than sulfhydryl groups at the active site because the enzyme was not inhibited by iodoacetate. Addition of PMSF-a protease inhibitor had no effect on amylase activity indicating final enzyme preparation to be protease free. Contrarily, Arikan (2008) reported that PMSF inhibited amylase activity from *Bacillus* sp. A3-15.

From the results, it is clear that the enzyme has the ability to work in a wider temperature and pH range and has high thermostability suggesting that it can be used for starch hydrolysis at temperature which restricts microbial growth. The reported pH value indicates it to be similar to those required for efficient starch liquefaction. Therefore, this enzyme can be used in industrial sector.

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Evaluation of *Pseudomonas fluorescens* for the biocontrol of fusarium wilt in tomato and flax

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Fusarium oxysporum **is an abundant plant pathogen in the soil and is found in the rhizosphere of many plant species. The fungus has numerous formae speciales (f.sp.) that infect and cause wilt diseases of a variety of host plants. *F. oxysporum* f.sp *lycopersici* (Fol) and *F. oxysporum* f.sp *lini* (Foln) are known to infect tomato and flax, respectively. Fluorescent pseudomonads can suppress various soilborne diseases, and their efficacy has been related both to their antagonistic activities and to their rhizosphere competitiveness. In this study, the biocontrol capacity of plant growth-promoting rhizobacteria (PGPR) strains of *Pseudomonas fluorescens* against fusarium wilt of tomato and flax was evaluated both *in vitro* and *in planta*. The *P. fluorescens* strains exhibited substantial antagonistic activities against the two fungal isolates on three culture media tested: King B (KB), potato dextrose agar (PDA) and a mixture of both (KB + PDA). As compared to the untreated control, the mycelial growth decreased by 8.33 to 49.33%. Conidial germination and germ tube elongation were significantly inhibited and reduced, respectively, by 6.15 to 47.33% and by 1.63 to 45.45%. In bioassay trials, *P. fluorescens* strains significantly reduced the disease incidence and severity by 4.86 to 74.49% on tomato plants and 3.93 to 79.19% on flax plants. The different efficacy of the biocontrol agents could be due to the influence of several factors, including the efficiency of the strain, the type of pathogen and the host.**

Key words: Plant growth-promoting rhizobacteria (PGPR), *Pseudomonas fluorescens*, fusarium wilt, tomato, flax, biocontrol.

INTRODUCTION

Disease suppression by biocontrol agents is the manifestation of interactions among the plant, pathogen, biocontrol agent, microbial community on and around the plant and physical environment. Among the wide range of beneficial microorganisms, plant growth-promoting rhizobacteria (PGPR) play a vital role in the management of plant diseases (Kloepper and Schroth, 1978; Weller, 1988; Keel et al., 1992; Haas and Defago, 2005; Siddiqui, 2006; Yan et al., 2009). In microbial interactions of the rhizosphere, telluric fluorescent pseudomonads are associated with various saprophytic and/or parasitic microbial populations, according to the host plant. *Fusarium oxysporum* Schlecht., a complex group that presents many formae speciales and physiological species, is an extremely common soil fungus that occurs in the rhizosphere of many plant species. The group is often dominant within the fusarium soil populations and causes vascular wilt that result in considerable economic losses (Armstrong and Armstrong, 1981).

Fusarium vascular wilt is a severe disease that can

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affect plants of different botanical families (e.g., tomato and flax), and for some of the causative agents, such as *F. oxysporum* f.sp *lycopersici* and *F oxysporum* f.sp *lini*, there are no effective control methods. *F. oxysporum* produces chlamydospores, macroconidia and microconidia, and all of these stages are present in infected tissues or soil (Nelson, 1981). The systemic nature and the infectious and epidemic character of the vascular fusarium wilts limit the use of possible preventative measures and conventional disease controls (Gordon and Martyn, 1997; Duijff et al., 1998; Larkin and Fravel, 1999; De Boer et al., 2003; Fravel et al., 2003). Despite the use of resistant cultivars, the occurrence and development of new pathogenic species is a continuous problem. Therefore, the application of fungicides is a normal practice; however, this may not be very effective because the disease appears late in the growth of the crop, and the persistence of fungicides throughout the crop cycle is always doubtful. The use of systemic fungicides has remained the method used most often, despite the risk of the emergence of resistant strains.

F. oxysporum species have been used extensively as models for their interactions with PGPR, and based on the antagonistic activities of the PGPR fluorescent pseudomonads, the microbiological control against *F. oxysporum* has been the object of extensive research carried out under both controlled and natural conditions (Bakker et al., 1990; Lemanceau and Alabouvette, 1993; Alabouvette et al., 2006). The fluorescent pseudomonads are considered potential biocontrol agents of plant telluric diseases, and several studies have shown their efficacy as an inoculum (Kloepper et al., 1980; Tomashow and Weller, 1995; Lugtenberg et al., 1999; Whipps, 2001; Weller et al., 2002; Achouak et al., 2004; Hariprasad; et al., 2009; Validov et al., 2009). Several PGPR formulations are currently available as commercial products for agricultural production of beneficial crops, such as BlightBan A506, Conquer and Victus formulated with *Pseudomonas fluorescens* strains, for the pratical use on crops of almond, apple, cherry, peach, pear, potato, strawberry and tomato (Chet and Chernin, 2002; Bhattacharyya and Jha, 2012).

These beneficial strains can colonize the soil, seeds, root surfaces and other underground parts of the plants to the same extent as the phytopathogens that they control (Keel et al., 1992; Whipps, 2001).

Significant results concerning the biocontrol of many plant diseases have been obtained using tobacco (Maurhofer et al., 1995), cereals (Duffy et al., 1997), and horticultural plants (Kloepper et al., 1992; Duijff et al., 1998; Fravel et al., 2003) involving numerous fusarium diseases (Keel et al., 1992; Raaijmakers et al., 1995; Latour et al., 1996; Leeman et al., 1996; Weller et al., 2002). Fluorescent pseudomonad bacteria have been shown to act against pathogenic agents by synthesizing antibiotic compounds (e.g., phenazins, pyrrolnitrine and 2,4- diacetylphloroglucinol) (Keel et al., 1992), hydrogen cyanide (Maurhofer et al., 1995), lytic enzymes capable of altering the fungal cell wall (chitinase and glucanase) and other secondary metabolites (O'Sullivan and O'Gara, 1992). In addition to the antibiotic properties and the trophic competition recognized in these rhizobacteria, there is evidence that fluorescent pseudomonad strains can trigger induced systemic resistance (ISR) in plants, thus assuring a protection against a broad spectrum of phytopathogen agents (Van Loon et al., 1998).

The potential for the biological control of fusarium wilt has attracted much attention throughout the world. At present, the idea of controlling soil-borne plant pathogens, including Fusaria, with chemical pesticides or fungicides is being challenged by the approach that biological control can have an important role in sustainable agriculture (Pandey et al., 2010).

The purpose of this study was to evaluate the antifungal activity, *in vitro* and *in planta,* of *P. fluorescens* strains against *F. oxysporum* f.sp *lycopersici* and f.sp *lini*, the causative agents of fusarium wilt of tomato and flax, respectively.

MATERIALS AND METHODS

Antagonistic bacteria

On the basis of their metabolic characteristics and their biocontrol potential, the PGPR strains used in this work were selected according to their origin and their ability to produce (or not) secondary metabolites *in vitro* that are implicit in biocontrol, namely, pyoverdines (pvd), hydrocyanic acid (HCN), indole acetic acid (IAA), phosphate-solubilizing compound (phos) and phenazins (phz) (Table 1).

Five *P. fluorescens* strains, originally isolated from the rhizosphere of different plants, were selected from several PGPR strains based on their efficiency as biocontrol agents, as demonstrated by significant reductions in root and foliar diseases following their application as a seed or soil treatment in tomato, wheat, flax and palm date (Benchabane, 2005) (Table 1). The *P. fluorescens* strains CHA0 and CHA400 were kindly supplied by K. Keel (Laboratoire de Biologie Microbienne, Université de Lausanne, Switzerland). CHA0 produces a variety of secondary metabolites, promotes the growth of various crop plants and protects plants against root diseases caused by soilborne pathogenic fungi (Keel et al., 1992; De Werra et al., 2009). The genetic derivative, CHA400, differs from the wild-type strain, CHA0, by the absence of pyoverdine synthesis (Muller, 2009). Bacteria were scraped from the surface of a 24-h old King's B (KB) agar plate (King et al., 1954) and resuspended in 50 ml sdH_2O (sterilized distilled water); the density was then adjusted to 10^6 cfu/ml using a spectrophotometer (Shimadzu-m240, λ= 620 nm). Each bacterial strain was stored at -80°C in Eppendorf tubes (200 µl of sterile glycerol + 800 µl of bacterial suspension in KB liquid medium).

Plant pathogens

The *F. oxysporum* f.sp *lycopersici* isolate (Fol) (Collection of Phytopathology Laboratory, Agroveterinary Sciences Faculty, Blida University, Algeria) had originally been isolated from the roots of wilted tomato plants (Benchabane, 2005). *F. oxysporum* f.sp. *lini* (Foln) was kindly provided by P. Lemanceau (UMR Microbiologie

Table 1. Origin of the *P. fluorescens* strains.

Strain	Origin of isolation	Observation
P6	Potato rhizosphere (Algeria)	pvd +, HCN+, IAA +, phos +, phz +
B3	Wheat rhizosphere (Algeria)	pvd +, HCN+, IAA -, phos +, phz +
T9	Tomato rhizosphere (Algeria)	pvd +, HCN-, IAA +, phos -, phz +
SN1	Uncultivated soil (Algeria)	pvd +, HCN-, IAA -, phos -, phz -
D2	Date palm rhizosphere (Algeria)	pvd +, HCN+, IAA -, phos +, phz +
CHA0	Tobacco rhizosphere (Switzerland)	Reference strain, used in biocontrol and synthesize various secondary metabolites (Keel et al., 1991)
CHA400	Mutant of CHA0	pvd -

+: Positive; -: negative; pvd: pyoverdines; HCN: hydrocyanic acid; IAA: indole acetic acid; phos: Phosphatase, phz: phenazins.

du Sol et de l'Environnement, INRA- Dijon - France). Initially, the fungal pathogens were stored as a microconidial suspension in 30 % glycerol at -80°C; for all of the experiments, the fungi were maintained on potato dextrose agar (PDA, Sigma) slants and stored at 4°C. The purity of the fungal isolates was verified by monosporal cultures on PDA plates, using microscopic observations of the microconidia and macroconidia in addition to the stability of the cultural characteristics (colony shape, coloration and morphometric dimensions). The conidial suspensions were prepared as described by Steinkellner et al. (2008) with some modifications: the PDA fungal culture plates (eight days old, incubated at 28°C) were flooded with 2 ml sdH_2O and scraped into 48 ml sdH_2O. The microconidia suspensions obtained were filtered through six layers of sterile cheesecloth to separate the mycelium from the conidia and adjusted by a hemocytometer to 10^6 microconidia/ml.

In vitro antagonism

Antagonistic activities between individual *P. fluorescens* strains and *F. oxysporum* isolates (Fol and Foln) were tested in three culture media, Potato Dextrose Agar (PDA), KB (King et al., 1954) and a KB + PDA mixture (1:1 v/v), by measuring the inhibition of the mycelial growth, conidial germination and germ tube elongation. Before use, the two fungus isolates were grown separately on PDA plates for 8 days at 28°C, and the bacteria isolates were grown separately in liquid KB medium for 24 h at 26°C.

Mycelial growth

Tests for antibiosis were conducted on solid culture media plates using the dual culture technique adapted as described by Keel et al. (1996). Each bacteria strain was spotted with a loopful culture (24 h old) on four corners of a Petri plate and incubated at 26°C. Twenty-four hours later, 5-mm diameter PDA plugs containing mycelia of Fol or Foln (5 days old) were placed in the center of the plates and incubated at the same temperature. Plates inoculated with only fungal agar plugs served as a control and were observed daily until their maximal growth (8 at 10 days). The fungal growth was measured in the dual culture plates, where the colony diameter was measured in three directions on each plate, and the percent inhibition was calculated in three independent replicates as follows:

([radial growth in control - radial growth in dual culture] / radial growth in control) x 100

Conidial germination and the elongation of the germ tube

Dual inoculations of suspensions of conidia and bacteria were prepared by adding 1 ml of bacterial suspension into test tubes containing 8 ml liquid medium (PD, KB or PD + KB). After 6 h of pre-incubation at 2 °C, 1 ml of conidial suspension was added and incubated for 18 h at the same temperature in the dark while being shaken at 100 rpm. Control treatments were prepared by substitution of the bacterial suspension by 1 ml sdH_2O. The experiments were performed five times. As described by Wu et al. (2009), six (40 µl) drops of the suspension for each replicate was placed on a glass slide and mixed with a drop of acid fuchsin in lactophenol to kill and stain the conidia. The mixture was observed with a light microscope (400x magnification) calibrated to determine conidial germination and the length of the germ tubes (µm) in six randomly chosen microscopic fields per glass (200 - 300 conidia x 6). A microconidium was considered germinated if the germ tube length was at least as long as the spore. For each field, the total number of conidia was counted, and the percentage of the inhibition of conidial germination and germ tube elongation was calculated as follows:

ICG = ([GC - GD] / GC) X 100,

where ICG is the inhibition of conidial germination (%), GC is the percentage of germination in the control and GD is the percentage of germination in the dual inoculation.

IGTE = ([LC - LD] / LC) X 100

Where, IGTE is the inhibition of germ tube elongation (%), LC is the length of the germ tubes in the control treatment and LD is the length of the germ tubes in the dual treatment.

In planta bioassay

The experiments were performed independently for testing the antagonistic activities *in planta (*tomato and flax) of *P. fluorescens* strains against Fol and Foln, respectively, in a greenhouse under a day/night cycle of 16/8 h, 28°C/18°C ± 2°C and 60 - 65% relative humidity.

Soil and culture substrate

The soil, loamy sand, pH 7.6, organic matter 2% (originally from the Experimental Station of the Department of Agronomy, Blida

Table 2. Disease scale of tomato and flax wilts.

Tomato wilt	Flax wilt
0 : Plant healthy	0 = Plant healthy
1 : Plant with foliar basal wilt	1 = Partial yellowing
2: Plant with unilateral wilt	2 = Apical wilting «Shephard's Crook»
3 : Plant completely wilted	3 = Partial wilting
4: Plant completely desiccated and dead	4 = Plant completely wilted
	5 = Plant completely desiccated and dead

University), had been cultivated regularly over the last five years with tomatoes. The soil was passed through a 3 mm sieve, air-dried and added to sterilized peat (2:1). The resulting substrate was autoclaved (120°C) for 1 h on two consecutive days and used to fill plastic pots (4.5 kg substrate/pot).

Pathogen inoculation and seed bacterization

The application of pathogens and antagonists was carried out using an adaptation of the procedures described by Validov et al. (2009). The soil in the pots was inoculated with the fungal pathogens (Fol or Foln, 10^6 conidia/g of soil) using the conidial suspension prepared above 48 h prior to the bacterization. For the untreated (control) soil, the same volume of conidial suspension was replaced by sdH_2O. Certified seeds of tomato (*Solanum lycopersicum* cv. riogrande) and flax (*Linum usitatissimum* cv. opaline) were surface sterilized (3% NaOCl) for 5 min, rinsed three times in sdH_2O and dried on absorbent paper. The bacterial suspension was prepared with methylcellulose (1 %) as an emulsifier and agitated for 2 h (100 rpm) prior to use.

The bacteria-methylcellulose suspension was mixed with either the tomato or flax seeds and then dried overnight in a laminar air flow hood and transferred onto moist filter paper (10^7 cfu/seed). The control treatments consisted of non-bacterized dry seeds or seeds only coated with methylcellulose (1 %). Three bacterized seeds were initially sown per pot, and after emergence (12 - 16 days after plantation), only one plant was kept; the bacterial suspension (10^7 cfu/ml) was applied as a soil drench around the plant base stem. The plants were irrigated as needed with sterile water.

Assessment of disease development

The disease incidence at each observation was calculated by the number of plants that showed symptoms of wilting as a percentage of the total number of plants of each treatment per block. Subsequently, the disease ratings were plotted over time to generate the disease progress curves. Symptoms of fusarium wilt disease were assessed every 3 days using the following two scales, corresponding to each disease according to the growth characteristics of each plant: (i) tomato disease (0 - 4) and (ii) flax disease (0 - 5) (Table 2).

The plant was considered infected when a rating of 2 was recorded. The disease incidence was noted by the calculation of the infection percentage (I%) and by the assessment of the disease severity (S%) as follows:

$$I\ (\%) = (\text{number of plants infected} / \text{total number of plants observed}) \times 100$$

$$S\ (\%) = (\Sigma\ [E.a] / N.T \bullet 100)$$

Where, E is the disease scale (0 to 4) for tomato wilt and (0 to 5) for flax wilt, a is the number of plants infected at each symptomatological scale, N is the total number of plants observed and T is the maximum disease scale (4 for tomato wilt and 5 for flax wilt).

The biological control was estimated on the basis of a comparative report between the levels of the diseases amongst the positive control treatment in relation to the bacterized treatments.

Experimental design

For the experiment, each trial (with Fol or with Foln) was conducted independently in a completely randomized block design (factorial) with four replicates (four random blocks). The studies are on nine treatments per block: seven treatments corresponding to the seven bacteria strains (P6, B3, T9, SN1, D2, CHA0 and CHA400) and two controls, a positive control T(+), inoculated only with fungal conidial suspension but not bacterized, and a negative control T(-) not bacterized and not inoculated by fungi. For each trial, at least 100 tomato plants and 100 flax plants were used (25 plants per block x 4). The same design was repeated twice for each trial under the same conditions.

Statistical analysis

Statistical analyses were performed with the SPSS software (version 16.0 for windows). The data obtained were subjected to analysis of variance (ANOVA).

For the *in planta* experiments, each treatment corresponded to the means of the total trials performed. The treatment means were compared and separated using the Newman-Keuls test on the basis of the little significant amplitude (LSA) by the F values ($P \leq 0.05$). The data for the relative inhibition of mycelial growth, conidial germination and germ tube elongation were square root transformed; the infection percentage and disease severity were arcsine square root transformed before analysis.

RESULTS

In vitro antagonism activities

Inhibition of mycelial growth

The five *P. fluorescens* strains exhibited substantial antagonistic activities against the two fungal isolates on the three culture media tested, causing clear inhibition zones in the dual cultures and restricting the growth of the two formae speciales of *F. oxysporum* by 8.33 to 49.33% (T9 - Foln). As compared to the untreated

Table 3. *In vitro* inhibition of mycelial growth (%) on the three culture media tested.

Bacterial strains	PDA		KB		PDA + KB	
	(Fol)	(Foln)	(Fol)	(Foln)	(Fol)	(Foln)
B3	31.39±1.69*[a]**	28.40±1.42[b]	38.52±1.83[a]	39.78±1.99[b]	28.50±1.89[a]	22.66±1.09[b]
P6	33.72±1.74[a]	39.95±2.39[a]	26.40±1.29[b]	29.95±1.51[c]	22.30±1.23[b]	31.15±1.42[a]
T9	34.88±0.74[a]	35.33±1.67[a]	34.33±1.85[a]	49.33±2.45[a]	24.33±1.70[b]	08.33±0.45[c]
D2	14.86±1.74[b]	24.57±2.28[c]	33.40±1.76[a]	30.19±1.51[c]	25.48±1.64[a]	25.33±1.45[b]
SN1	36.75±1.14[a]	33.52±1.86[a]	36.25±1.79[a]	23.50±2.23[d]	31.18±1.78[a]	33.88±1.86[a]

Fol: *Fusarium oxysporum* f.sp. *lycopersici*; Foln: *Fusarium oxysporum* f.sp. *lini.**: Standard deviation; **: The values followed by the same letter are not significantly different according to the Newman-Keuls test (P < 5%).

control, the mycelial growth decreased for *F. oxysporum* f.sp. *lycopersici* (Fol) by 14.86 to 36.75% on PDA, 26.40 to 38.52% on KB and 22.30 to 31.18% on KB + PD media, whereas the mycelial growth of *F. oxysporum* f.sp. *lini* (Foln) was reduced by 28.40 to 39.95% on PDA, 23.50 to 49.35% on KB and 8.33 to 33.88% on KB+PD. On the basis of the diameter of the inhibition zone, the strains were separated further into two to four groups (a, b, c or d) according to their interactions, but the level of inhibition remained statistically significant. Strain T9 exhibited more variability, with a maximal inhibition value against Foln, yet gave the minimum value against the same pathogen on KB + PDA medium (Table 3). Due to this exception, the effect of the culture media was noted but was not apparent in other interactions.

Inhibition of conidial germination and germ tube elongation

Conidial germination and germ tube elongation were significantly inhibited by the *P. fluorescens* strains. The conidial germination percentage was reduced by 9.34 to 55.95% (Fol) and by 6.15 to 29.38% (Foln). The germ tube elongation percentage was reduced by 5.42 to 45.45% (Fol) and by 1.63 to 20.89% (Foln). The antagonistic activities of the tested PGPR strains varied with respect to the target isolates of *F. oxysporum*, but in general, they were more important against Fol than Foln. The inhibition level noted with strains B3, P6 and SN1 were stronger in KB medium than in the two other media tested, whereas they were higher in PD medium for the T9 and D2 strains. For the two estimated parameters (IGC and IETG), the T9 strain resulted in the highest rates of inhibition of conidial germination and germ tube elongation against Fol (Table 4).

In planta bioassay

Seven bacterial strains with notable antagonistic activities were selected for evaluation in the bioassay trials. In the pathogen control (T+) with the SN1, B3, T9 and CHA400 treatments, disease symptoms were visible from the 10th day for tomato (Figure 1) and after the 15th day for flax (Figure 2). After inoculation of the bacterial strains, the wilt disease reached considerable levels by the 17th (tomato) and 25th (flax) day, yet the plants showed a reduction in the evolution of the degree of the disease; in the control, the rate of infection rose rapidly after 17 days (> 50 %).

At the end of the two experiments, analysis of variance showed significant differences between treatments (P < 0.05), and the Newman-Keuls test differentiated five groups (tomato wilt) and six groups (flax wilt) that differed significantly from each other (Figures 1 and 2). None of the bacterial strains completely protected the plants, although all of the isolates significantly decreased the severity and incidence of the disease.

At the end of our experiments, the severity of the disease on the tomato and flax plants was reduced to different levels depending on the bacterial strain and was estimated to be 4.86 to 74.49% on the tomato plants and 3.93 to 79.19% on the flax plants (Figure 3). Similar decreases in wilt disease were observed with strains B3, D2 and CHA0 (the same Newman-Keuls group) against Fol and Foln.

The high biocontrol effects (> 60%) of the CHA400 and P6 strains were classed into two groups (Figure 2). The least amount of inhibition was noted with SN1 against the two diseases and the T9 strain against tomato fusarium wilt (Figure 3).

DISCUSSION

The use of beneficial microorganisms as biopesticides to reduce diseases on various agronomically important crops is considered one of the most promising methods in crop management practices. In the present study, we evaluated the efficacy, *in vivo* and *in planta*, of *P. fluorescens* strains as biocontrol agents against *F. oxysporum* f.sp *lycopersici* and *F. o* f.sp. *lini*.

Despite the difference in the chemical composition of the medium and its influence on the synthesis of metabolites, which can have a major function in the

Table 4. *In vitro* inhibition of conidial germination and germ tube elongation (%) on the three culture media tested.

Bacterial strain	Media	ICG (%)		IGTE (%)	
		Fol	(Foln)	Fol	Foln
B3	KB	22.10±3.58*^{c}	11.33±2.08^{d}	26.02±7.58^{bc}	06.28±0.08^{e}
	PD	18.26±1.54^{c}	13.44±1.06^{d}	19.36±1.28^{c}	06.44±0.06^{e}
	PD+KB	09.34±0.65^{d}	16.25±1.65^{c}	05.42±0.65^{e}	09.43±0.65^{de}
P6	KB	37.33±2.90^{b}	29.38±1.95^{bc}	26.62±1.82^{bc}	20.89±1.63^{c}
	PD	16.26±3.14^{c}	12.35±2.11^{d}	09.58±0.72^{de}	10.58±1.18^{d}
	PD+KB	16.58±2.78^{c}	26.35±2.75^{bc}	10.52±1.11^{d}	10.36±0.31^{d}
T9	KB	47.33±5.85^{a}	15.33±1.22^{d}	42.04±4.26^{a}	08.38±3.82^{de}
	PD	55.95±6.52^{a}	14.69±3.52^{d}	44.97±5.85^{a}	07.16±1.52^{e}
	PD+KB	17.88±2.26^{c}	06.17±3.52^{e}	11.12±2.45^{d}	02.29±0.07f
D2	KB	23.21±3.22^{c}	13.17±2.33^{e}	09.12±0.82^{de}	09.15±1.28^{de}
	PD	46.65±6.18^{a}	14.69±2.92^{d}	45.45±1.51^{a}	08.11±1.32^{de}
	PD+KB	18.69±2.42^{c}	06.15±1.52^{e}	13.42±1.38^{d}	01.63±0.05f
SN1	KB	22.31±3.33^{c}	13.15±1.56^{d}	11.11±3.33^{d}	07.34±1.12^{e}
	PD	16.26±1.14^{c}	12.35±2.11^{d}	08.25±0.74^{de}	05.28±0.75^{e}
	PD+KB	16.58±1.78^{c}	26.35±2.35^{bc}	08.38±1.08^{de}	16.32±0.85^{c}

ICG: Inhibition of conidial germination; IGTE: inhibition of germ tubes elongation; Fol*: Fusarium oxysporum* f.sp. *lycopersici*; Foln: *Fusarium oxysporum* f.sp. *lini.* *Standard deviation. The values followed by the same letter are not significantly different according to the Newman-Keuls test (P < 5%).

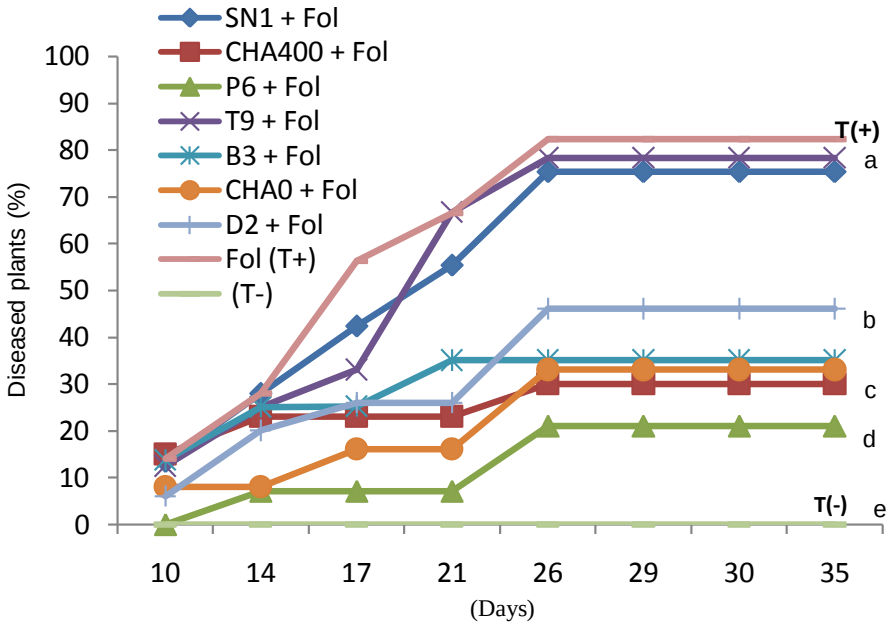

Figure 1. Disease evolution on tomato plants. The treatments followed by the same letter are not significantly different according to the Newman-Keuls test (P < 0.05). *P. fluorescens* strains: SN1, CHA400, P6, T9; B3, CHA0, D2. Fol (T+) : Positive control T(+), inoculated only with fungal conidial suspension but not bacterized. (T-): Negative control; T(-) not bacterized and not inoculated by fungi; Fol *: Fusarium oxysporum* f.sp. *lycopersici*.

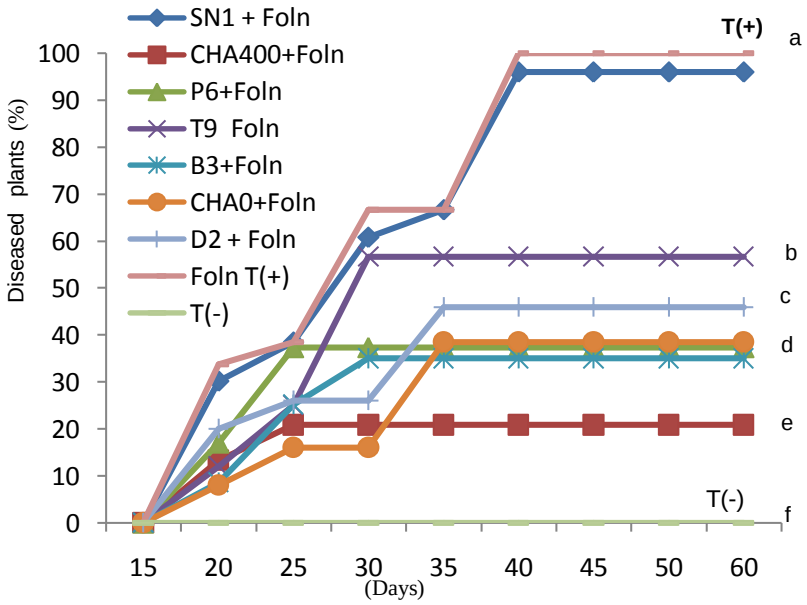

Figure 2. Disease evolution on flax plants. The treatments followed by the same letter are not significantly different according to the Newman-Keuls test ($P < 0.05$). *P. fluorescens* strains: SN1, CHA400, P6, T9, B3, CHA0, D2. Foln T(+) : Positive control, inoculated only with fungal conidial suspension but not bacterized. T(-) : Negative control, not bacterized and not inoculated by fungi. Foln : *Fusarium oxysporum* f.sp. *lini.*

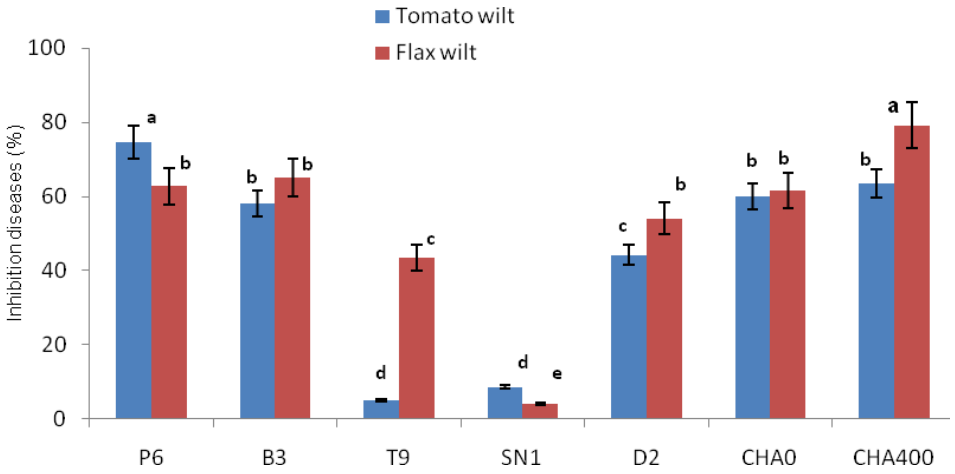

Figure 3. Biological protection rates against fusarium wilt of tomato and flax. Different upper-case letters indicated the difference in statistics according to the Newman-Keuls test ($P < 0.05$). *Pseudomonas fluorescens* strains: SN1, CHA400, P6, T9, B3, CHA0, D2.

inhibition effect; the three utilized culture media permitted the observation of antagonism for all of the five tested bacterial strains. We noted that the KB medium favored the bacterial synthesis of siderophores, whereas the PD and PD + KB media did not. The inhibition of the conidial germination and the elongation of the germ tubes observed on these two media suggested that the observed inhibition was not due to the action of the siderophores but that other mechanisms were developed by the bacteria, unlike the conidial germination inhibited on the KB media, which can be correlated with the presence of the siderophores.

It has been previously demonstrated that the antagonistic action of these same strains did not seem to be specific for the pathogenic agent but rather depended on the culture medium; in some cases, a broad-spectrum efficacy has been observed, acting at the same time on several fungal isolates of different genera (*Fusarium*,

Rhizoctonia, *Vertcillium* and *Pythium*) (Benchabane, 2005). The variability in the antagonistic activity of the *Pseudomonas* strains in the media tested suggested a diversity in the mechanisms implied in the biocontrol. In addition to the plurifactorial determinism of the antagonism, our results underline the possibility of a broad, non-specific efficacy against the two isolates of *F. oxysporum* (Fol and Foln). The difference in mycelial stimulation or inhibition in culture media results from physiological mechanisms under the culture conditions. Whereas spores are critical to the spatial dispersal of fungi, spore germination is essential to their survival and the establishment of an actively growing colony on suitable substrates. Invasive germ tube growth, such as the typical phenotype of *Fusarium* species, during surface germination is anomalous and unique to a small collection of strains or certain species (Glenn, 2006).

The signals affecting spore germination of the tomato pathogen, *F. oxysporum*, do not seem to be host-specific, as a similar pattern of microconidial germination has been found in the presence of root exudates from the host tomato plant and several non-host plants (sweet pepper, bean, barley, tobacco and cucumber). However, there are indications for a specific effect of the compounds found in tomato root exudates, and it has been reported that the germination rate of several *F. oxysporum* strains is affected differently by tomato root exudates (Steinkellner et al., 2008). No differences in the mycelial development of *F. oxysporum* have been observed in the vicinity of tomato and wheat roots, such that non-host plants can function as symptomless carriers of fusarium wilt (Steinberg et al., 1999). Perhaps the observed variation can be attributed to the biological variation of the fungal strains and/or cellular structures. However, further studies are needed to characterize the signaling compounds in plant root exudates that may be involved in the host plant interactions with F*. oxysporum*. Because spore developmental processes are critical for the reproduction and survival of fungi, spore germination for a plant pathogenic fungus may be seen as a critical developmental step in pathogenesis: germination must occur at both the correct time and location in order for infection to be successful (Seong et al., 2008).

The seven *P. fluorescens* strains used in our experiments showed varying antagonistic effects against both Fol and Foln. Theses rhizobacterial strains are characterized by different potentialities in terms of the production of metabolites implicated in biocontrol, such as the synthesis of pyoverdine (except CHA400) and the variation (+ or -) for other characteristics, especially the synthesis of phenazins and HCN production (Benchabane, 2005) (Table 1). When compared with wild type, the genetic derivative, CHA400, exhibits similar inhibition levels, although this strain lacks the ability to produce the strong siderophore pyoverdine (Keel et al., 1992), and therefore, should not take up iron. In contrast, the wild type and various mutants of the CHA0 strain complex iron by producing different siderophores, namely, pyochelin (Ankenbauer et al., 1988), salicylic acid (Meyer et al., 1992) and pyoverdine (Albrecht-Gary et al., 1994), which all form strong complexes with Fe (Müller, 2009).

The infection process of *F. oxysporum* involves the following steps: germination of spores in response to root exudates, production of penetration hyphae that attach to the root surface and directly penetrate it, and invasive growth in the host plant tissue (Rodríguez-Gálvez and Mendgen, 1995). The stimulation of plant pathogens by root exudates is an integral part of the concept of inoculum potential (Lockwood, 1986), and extensive studies have been performed on the biology of *F. oxysporum* and the colonization process (Steinkellner et al., 2008). Root exudates are supposed to play a key role in determining the positive or negative outcome of an interaction in the rhizosphere by various metabolites that are known as general germination stimuli for spores (Nelson, 1991; Bais et al., 2006). At different growth stages, tomato roots exude varying amounts of sugars and organic acids, and depending on the plant age, the effect of the tomato root exudates on spore germination of the tomato pathogen *F. oxysporum* also varies (Lugtenberg et al.,1999; Steinkellner et al., 2008).

In this study, overall, the most active rhizobacteria strains were P6, B3 and T9, and their antagonistic activities can be correlated with their metabolic characters, notably their secondary metabolites (phenazins) (Benchabane, 2005). The inefficiency of the T9 and D2 strains against *F. oxysporum* f.sp *lycopersici* reinforces their absence in relation to their rhizospherical origin and their antagonist activities. However, the lack or absence of an antagonistic effect for the SN1 strain, isolated in non-rhizospherical soil, showed that, independent of the biotope around the plant, a rhizospherical origin seems to play a primary role in biocontrol and appears to have a correlation with their capacity of rhizospherical adaptation and their acquired competitiveness. However, a rhizospherical origin of the bacterial strains did not seem to play a role in their *in vitro* antagonistic activities; thus, the T9 and D2 strains isolated from tomatoes and palm date rhizospheres, respectively, did not present a specific and regular antagonism against the *F. oxysporum* isolates. Two types of disease suppression are usually distinguished: (i) specific disease suppression (Weller et al., 2002) is caused by one or a few specific (genotypes of) microorganisms, such as phloroglucinol-producing pseudomonads suppressing *Gaeumannomyces graminis* (Raaijmakers and Weller, 1998), and (ii) general disease suppression is caused by multiple microorganisms acting against multiple pathogens and is restored quickly after a major disturbance (Hoitink and Boehm, 1999). The occurrence of specifically acting antagonists can occur anywhere but seems to be most dominant in the rhizosphere soil and, thus, is not entirely congruent with pathogen suppression (Termorshuizen and Jeger, 2008).

The growth and activity of the root system induces

significant modifications in the physicochemical and biological properties of the soil surrounding the root (rhizosphere effect) due to rhizodeposition. The ability to support certain biocontrol agents varies among plant species, cultivars and genotypes (Lucy et al., 2004). The secretion of rhizodeposition is an important way for plants to respond to their environment, and root exudates mediate communication between plants and other organisms (pathogens and antagonists) and stimulate defense responses against soil-borne pathogens and/or favor the association with beneficial soil microbes. The plant species, cultivar and phenological stage also have a primary role in the modulation of the quantity and the quality of the root exudates, similar to the physiological reactions to stress factors during the development microorganisms in the rhizosphere and their adhesion on root compounds (Nelson, 1991; Lugtenberg et al., 1999).

The results from the plant (seeds) that were bacterized with *P. fluorescens* strains showed varying degrees of bioprotection that were invoked in flax and tomato, as well as other plants (Kloepper et al., 1980; Maurhofer et al., 1995; Raaijmakers and Weller, 1998). PGPR antagonize plant pathogenic fungi, mainly by the production of antimicrobial metabolites but also by the competition for iron or rhizosphere niches (Keel et al., 1992) and the stimulation of the host defenses (induced systemic resistance) (Van Loon et al., 1998). Other mechanisms are involved directly in the promotion of plant growth and modulate the biocontrol activity of the bacteria (Lemanceau and Alabouvette, 1993; De Werra et al., 2009).

In conclusion, our results show that *P. fluorescens* strains have great potential to be used as biocontrol agents for the management of the *Fusarium* species that cause fusarium wilt of tomato and flax. The *in vitro* and *in vivo* data support the hypothesis that antagonism is the main mechanism for the biological control of disease; however, the antimicrobial compounds responsible have not yet been isolated and identified.

Thus, further study is necessary for determining the mechanisms of antago-nistic action of these strains. Our results show that the *Pseudomonas* strains, selected on the basis of their recognized genetic, physiological, metabolic and ecological properties of antagonism, can constitute an efficient biological control.

The non-specific action of the bacterial strains against phytopathogenic fungi, the variability of the responses according to the environment and the diversity of the implicated mechanisms (and even redundant in some their action should be considered when selecting strains. PGPR have great potential in both phytostimulation and the biocontrol of plant pathogens, but they have not been widely applied in the field for various reasons, such as the problem of formulation for efficient application (Akhtar and Sidiqui, 2009).

Through the overlap of a number of characteristics for antagonism, the selection of candidates (bacterial strains) with different mechanisms of action should reinforce the final biocontrol effect. Furthermore, the association (co-inoculation) of different strains characterized by a variety of antagonistic features may result in a synergistic effect (or at least an additive effect) and a broader spectrum of action.

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Full Length Research Paper

Genetic diversity of iturin producing strains of *Bacillus* species antagonistic to *Ralstonia solanacerarum* causing bacterial wilt disease in tomato

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***Bacillus* spp. is a potential bacterial antagonist to manage bacterial wilt disease of tomato incited by *Ralstonia solanacearum,* which is one of the most threatening diseases of tomato in India. Genetic diversity of *Bacillus* strains and their potentiality to control bacterial wilt of tomato isolated from rhizospheric soil and endophytic tomato plants from different agro-climatic regions of India were studied. Rhizospheric soil and plants of tomato were pasteurized at 80°C for 15 min before dilution and then inoculated onto the Petri plates containing tryptic soy agar medium and incubated at 28± 2°C. Out of 250 isolates of *Bacillus* species, 47 strains showed antagonistic ability against *R. solanacearum.* Maximum growth of *R. solanacearum* was inhibited by strain DTBS-5 to form inhibition zone of 5.5 cm^2 *in vitro* and lowest wilt incidence of 14.3 and 7.6 % in Pusa Ruby and Arka Abha cultivars under glass house conditions, respectively. Plants treated with strain JTBS-9 had maximum fresh weight of 42.0 and 49.0 g and dry weight 6.1 and 6.6 g in tomato cultivars Pusa Ruby and Arka Abha after 45 days of transplanting, respectively. Out of 47 strains, 11 strains of *Bacillus* spp. were detected as iturin antibiotic producing strains by using iturin gene based marker. Genetic variability was found in *Bacillus* spp., which was made 5 clusters at 50% similarity coefficient. However, iturin producing and iturin non-producing strains as well as rhizospheric and endophytic *Bacillus* spp. could not be distinguished by using 16S rRNA sequence analysis and genetic fingerprinting.**

Key words: BOX-PCR, *Bacillus* spp., iturin, *Ralstonia solanacearum*, phylogenetic analysis, tomato.

INTRODUCTION

Bacterial wilt of tomato caused by *Ralstonia solanacearum* is one of the most devastating and wide spread diseases of crops worldwide (Hayward, 1991). In India, *R. solanacearum* race 1 biovar 3 is dominated mostly in coastal and hilly and foot hill area including Goa, Karnataka, Kerala, Maharashtra, Orissa, Jharkhand, West Bengal and state of North eastern hills, like Himachal Pradesh, Jammu and Kashmir and Uttarakhand (Devi and Menon, 1980). This disease causes very heavy loss varying from 2 - 90% in different climates and seasons in India (Mishra et al., 1995).

Management of this disease is very difficult due to soil borne nature of the pathogen, its wide host range of 200 species and 50 families including tomato, potato, egg

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plant, pepper, ground nut, tobacco, weeds and also roots of non host plants (Hayward, 1991; Poussier et al., 1999). There are no such chemicals available for effective management of this disease. Moreover, it causes soil and water pollution due to pesticide residues, which affects human health as well as development of resistant mutant by pathogen against pesticides. Hence, non-chemical methods including cultural methods, resistant cultivars and biocontrol with antagonistic bacterial agents have been made successfully to manage bacterial diseases of plants (Marten et al., 2000; Almoneafy et al., 2012). However, resistant cultivar is not completely effective due to lack of stability or durability (Boucher et al., 1992).

In biocontrol method, various fungal and bacterial antagonists were used to control plant diseases successfully. Among these bioagents, *Bacillus* species, a endospore forming, Gram positive bacterium is now a good candidate as a biocontrol agent and plant growth promoting bacteria (Chung et al., 2008). *Bacillus* spp. are able to colonize rhizosphere of plants and endophytic to form biofilm, which improve their ability to act as a bioagents against plant pathogens (Timmusk et al., 2005). Biological control of *R. solanacearum* through the antagonistic bacteria was done, which reduced the incidence of bacterial wilt disease (Almoneafy et al., 2012; Singh et al., 2012). The bacterium is found quite effective to suppress the bacterial pathogen (Rajendran et al., 2012; Singh et al., 2012) due to well developed secretary system and produces structurally diverse secondary metabolites with a wide spectrum of antibiotic activity. The *Bacillus subtilis* group was mostly able to synthesize surfactin and arthrobacin (Wulff et al., 2002). Chung et al. (2008) screened *B. subtilis* through PCR based on gene involved in biosynthesis of 11 antibiotics produced by various isolates of *B. subtilis.* Beside antagonistic ability, this bacterium can either directly or indirectly facilitate rooting and growth of plants by producing plant growth promoting substances like indole acetic acid, phosphate solubilization, ammonia and siderophore(Chaiharn et al., 2009).

Characterization of bacteria by using classical methods as morphological, biochemical, physiological is very laborious, time consuming. Hence, various advanced DNA based techniques are used to distinguish bacteria at species and sub species level. However, a molecular marker based on 16S rRNA sequence was developed to differentiate *Bacillus* species (Wattiau et al., 2001). Genetic diversity analysis of bacteria is studied by using BOX-PCR (Martin et al., 1992) and sequence analysis of 16S rRNA (Marten et al., 2000). These techniques have been successfully applied to *Bacillus* species for investigations of the relationships among isolates of *Bacillus* (Almoneafy et al., 2012). A little information is available on distinguishing antagonistic and plant growth promoting strains of *Bacillus* species by using genomic fingerprinting.

Thus, the present study was done to study genetic diversity of iturin producing strains of *Bacillus* species collected from different agro climatic conditions having antagonistic ability to suppress *R. solanacearum* and determine plant growth promoting attributes *in-vitro and vivo*.

MATERIALS AND METHODS

Sample collection

Rhizospheric soil and tomato plants were collected from 2009 - 2011 from different agro-climatic regions of India *viz.* Delhi, Jammu and Kashmir, Jharkhand, Himachal Pradesh, Manipur, Meghalaya, Uttarakhand and Uttar Pradesh states (Table 1). Physiochemical parameters of soil were analyzed and most of soils have electrical conductivity (EC) > 8.5, pH ranging from 6.0 - 8.5, sandy - loam to laterite soil (red soil). The plants were carefully removed from the soil and the whole plants with adherent soil were kept in plastic bags for isolation of bacteria.

Isolation of *Bacillus* from tomato plant and rhizosphere soil

For isolation of bacteria from rhizosphere soil, roots of tomato plants were shaken to remove excess soil and 10 g of rhizospheric soil from each sample was added to 90 ml of sterile distilled water and shaken for 30 min on a rotary shaker at 150 rpm. Soil samples were pasteurized at 80°C for 15 min for specific isolation of *Bacillus* spp. and then aliquots were further diluted up to 10^{-5}. The 100 µl aliquot of 10^{-3} and 10^{-5} dilutions were placed on to a tryptic soy agar (TSA) medium (Singh et al., 2012). The Petri plates were incubated at 28±1°C for 48 h.

For endophytic isolation of *Bacillus* spp., individual tomato plants were isolated as described by Zinniel et al. (2002) with slight modification as pasteurization at 80°C for 15 min. The single colony of bacterium was picked up and cultures were maintained on the TSA slants and stored at 4°C for further use.

Morphological, physiological and biochemical characters

Morphological and biochemical characterization of 44 isolates of *Bacillus* spp. was done based on the Gram reaction, KOH test, shape, motility, endospore formation and growth at different temperatures and NaCl concentrations. Biochemical characters like starch hydrolysis, utilization of citrate, indole production and H_2S production were done by using standard procedure (Schaad et al., 2001). Prototype strains used in taxonomic comparison were obtained from Institute of Microbial Technology, Chandigarh, India and Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi, India.

Antagonistic properties against *R. solanacearum*

Dual culture method was used for the screening of antagonistic properties of bacteria against *R. solanacearum* strain UTT -25 of *R. solanacerarum* was isolated from wilted tomato plant from District Nainital, Uttarakhand on TTC medium in 2009 as per standard procedure (Schaad et al., 2001). The *R. solanacearum* was grown in CPG broth medium for 48 h at 28 ±1°C and maintained the population of bacteria 10^8 cfu/ml. 100 µl culture of *R. solanacearum* was spread onto the Petri plates containing CPG medium to make a lawn of bacteria. Then 0.5 cm diameter of three wells in each Petri plate was made with sterilized cork borer. 40 µl of 48 h old

Table 1. Evaluation of antagonistic and plant growth promoting *Bacillus* species isolated from rhizospheric and endophytic of tomato from different agro-climatic conditions of India.

S/N	Strain of *Bacillus* spp.	Source	Location of collection	Area of inhibition zone against *R. solanacearum* (cm^2)	16SrRNA amplification	Iturin production	Phosphorus solubilization (µg/ ml)	IAA production (µg/ ml)	Siderophore production (µg/ ml)
1	MTBS-1	Rhizosphere	Nambol, Bishnupur, Manipur	1.2^{efgh}	+	-	34.3^{pq}	74.8^{cd}	0.63^{ijkl}
2	MTBS-2	,,	Nambol, Bishnupur, Manipur	1.1^{efgh}	+	-	30.3^{rs}	74.8^{cd}	0.67^{hijkl}
3	MTBE-1	Endophytic	Nambol, Bishnupur, Manipur	1.2^{efgh}	+	-	$44.7^{ijklmno}$	55.5^{cd}	0.90^{def}
4	MTBE-2	,,	Nambol, Bishnupur, Manipur	1.3^{defgh}	+	-	48.3^{ghi}	48.5^{cd}	0.73^{fghijk}
5	MTBS-3	Rhizosphere	Heikujm, Imphal west, Manipur	1.3^{defgh}	+	-	34.0^{pq}	48.5^{cd}	0.87^{defg}
6	MTBS-4	,,	Heikujm, Imphal west, Manipur	1.6^{defgh}	+	-	26.0^{tu}	55.46^{cd}	0.83^{defgh}
7	MTBE-3	Endophytic	Heikujm, Imphal west, Manipur	1.6^{defgh}	+	-	24.0^{u}	48.5^{cd}	1.0^{bcd}
8	MTBE-4	,,	Heikujm, Imphal west, Manipur	1.6^{defgh}	+	-	53.7^{f}	55.5^{cd}	0.77^{efghij}
9	MTBS-5	Rhizosphere	Wabagai, Thoubal, Manipur	0.8^{fgh}	+	-	66.7^{bc}	41.0^{d}	0.80^{efghi}
10	MTBS-6	,,	Wabagai, Thoubal, Manipur	1.3^{defgh}	+	-	66.7^{bc}	$41.^{d}$	0.70^{ghijkl}
11	MTBE-5	Endophytic	Wabagai, Thoubal, Manipur	1.0^{efgh}	+	-	42.3^{mno}	55.5^{cd}	0.57^{kl}
12	MTBE-6	,,	Wabagai, Thoubal, Manipur	1.6^{defgh}	+	-	41.3^{o}	41.0^{d}	0.93^{cde}
13	UTTBS-1	Rhizosphere	Khandi,Nainital, Uttarakhand	1.4^{defgh}	+	+	65.0^{cd}	55.5^{cd}	0.77^{efghij}
14	JHTBS-2	,,	Pithoria, Ranchi, Jharkhand	1.0^{efgh}	+	+	42.0^{no}	254.3^{bc}	0.87^{defg}
15	UTTBS-3	,,	Tihari, Nainital, Uttarakhand	1.3^{defgh}	+	+	42.3^{mno}	90.0^{bcd}	1.30^{a}
16	HPTBS-4	,,	Nagav, Solan, Himachal Pradesh	1.0^{efgh}	+	-	69.0b	55.5^{cd}	1.17^{ab}
17	JHTBS-5	,,	Pithoria, Ranchi, Jharkhand	1.5^{defgh}	+	+	60.7e	254.3^{bcd}	1.10^{bc}
18	JHTBS-6	,,	B A U, Ranchi, Jharkhand	1.2^{defgh}	+	-	46.3^{hijkl}	48.5^{cd}	1.13^{ab}
19	JHTBS-7	,,	B A U, Ranchi, Jharkhand	1.9^{def}	+	-	47.0^{ghij}	55.5^{cd}	0.87^{defg}
20	JHTBS-8	,,	ICAR Research Complex for Eastern Region, Palandu, Ranchi, Jharkhand	1.8^{defg}	+	+	41.3^{o}	55.5^{cd}	0.73^{fghijk}
21	JHTBS-9	,,	ICAR Research Complex for Eastern Region, Palandu, Ranchi, Jharkhand	1.9^{cde}	+	-	41.0^{o}	48.5^{cd}	0.57^{kl}
22	DTBS-4	Rhizosphere	Vegetable Farm, IARI, New Delhi	1.3^{defgh}	+	+	36.7^{p}	55.5^{d}	0.57^{kl}
23	DTBS-5	,,	Vegetable Farm, IARI, New Delhi	5.5^{a}	+	_	31.0^{qr}	48.5^{cd}	0.77^{efghij}
24	DTBS-6	,,	Vegetable Farm, IARI, New Delhi	1.9^{cde}	+	-	29.0^{rst}	48.5^{cd}	0.90^{def}
25	DTBE- 8	Endophytic	Vegetable Farm, IARI, New Delhi	1.8^{def}	+	-	32.0^{qr}	55.5^{cd}	0.63^{ijkl}
26	DTBE-9	,,	Vegetable Farm, IARI, New Delhi	1.5^{defgh}	+	-	36.7^{p}	90.1^{bcd}	1.30^{a}
27	JTBS-9	Rhizosphere	KVK, Kathua, Jammu & Kashmir	1.7^{defg}	+	-	72.7^{a}	55.5^{cd}	0.90^{def}
28	JTBE-14	Endophytic	KVK, Kathua, Jammu & Kashmir	2.1^{cde}	+	-	24.0^{u}	55.5^{cd}	0.77^{efghij}
29	JTBS-17	Rhizosphere	Basht, Udham Nagar, Jammu & Kashmir	1.5^{defgh}	+	-	24.7^{u}	48.5^{cd}	0.73^{fghijk}

Table 1. Contd

30	JTBE-20	Endophytic	Basht, Udham Nagar, Jammu & Kashmir	2.1^{cde}	+	-	31.0^{qr}	141.4^{a}	0.60^{jkl}
31	JTBS-21	Rhizosphere	S. K. University of Agriculture and Technology, Jammu, Jammu & Kashmir	1.5^{defgh}	+	-	29.3^{rst}	48.5^{cd}	0.73^{fghijk}
32	JTBE-23	Endophytic	S. K. University of Agriculture and Technology, Jammu, Jammu & Kashmir	1.0^{efgh}	+	-	$45.3^{hijklmn}$	48.5^{cd}	0.63^{ijkl}
33	UTBS-24	Rhizosphere	Naini, Allahabad, Uttar Pradesh	2.1^{cde}	+	-	62.3^{de}	48.5^{cd}	0.83^{defgh}
34	UTBE-25	Endophytic	Naini, Allahabad, Uttar Pradesh	0.5^{h}	+		63.7^{cde}	55.5^{cd}	0.73^{fghijk}
35	UTBE-26	,,	Naini, Allahabad, Uttar Pradesh	2.4^{cd}	+	-	64.3^{cd}	48.5^{cd}	0.83^{defgh}
36	UTBS- 32	Rhizosphere	SardarBallabhbhaiAgricutural University, Modipuram, Mereet, Uttar Pradesh	1.1^{efgh}	+	-	46.7^{hijk}	55.5^{cd}	0.57^{kl}
37	UTBS-33	,,	SardarBallabhbhaiAgricutural University, Modipuram, Mereet, Uttar Pradesh	0.7^{gh}	+	-	46.0^{hijklm}	288.5^{b}	0.83^{defgh}
38	UTBE-29	Endophytic	SardarBallabhbhaiAgricutural University, Modipuram, Mereet, Uttar Pradesh	2.0^{cde}	+	-	42.7^{lmn}	48.5^{cd}	0.70^{ghijkl}
39	UTBS-30	Rhizosphere	N. D. University of Agriculture &Technology, Faizabad, Uttar Pradesh	1.9^{cdef}	+	-	$45.3^{hijklmn}$	48.5^{cd}	0.80^{efghi}
40	UTBE-30	Endophytic	N. D. University of Agriculture &Technology, Faizabad, Uttar Pradesh	2.9^{bc}	+	-	49.0^{gh}	41.0^{d}	0.60^{jkl}
41	UTBE-31	,,	N. D. University of Agriculture &Technology, Faizabad, Uttar Pradesh	1.7^{defg}	+	-	50.3^{g}	48.5^{cd}	0.73^{fghijk}
42	METBS-31	Rhizosphere	ICAR, Research Complex, for NEH Region, Shilong, Meghalaya	3.6^{b}	+	-	41.7^{no}	48.5^{cd}	0.77^{efghij}
43	METBS-32	,,	ICAR, Research Complex, for NEH Region, Shilong, Meghalaya	2.1^{cde}	+	+	41.3^{o}	40.5^{cd}	0.93^{cde}
44	METBE-35	Endophytic	ICAR, Research Complex, NEH Region, Shilong, Meghalaya	3.4^{b}	+	+	43.0^{klmno}	55.5^{cd}	0.90^{def}
45	*B. Subtilis*	MTCC -2387	Institute of Microbial Technology, Chandigarh	1.5^{defgh}	+	+	43.7^{jklmno}	48.5^{cd}	0.73^{fghijk}
46	*B. cereus*	MTCC -7278	Institute of Microbial Technology, Chandigarh	1.7^{defg}	+	+	46.6^{hijk}	55.5^{cd}	0.87^{defg}
47.	*B. licheniformis*	-	Division of Plant Pathology, IARI, New Delhi- 110012	1.6^{defgh}	+	+	$45.3^{hijklmn}$	48.5^{cd}	0.77^{efghij}

+ = Positive reaction; - = negative reaction; Means followed by the same letter within a column are not significantly different as determined by LSD test (α = 0.05). Data present means of the experiment with 3 replications each.

culture of 250 isolates of *Bacillus* spp. including *B. cereus, B. licheniformis* and *B. subtilis* grown in the TS broth containing 10^9 cfu/ ml were poured into each well separately. The plates were incubated at 28 ±1°C for 24 h and inhibition zone formed by these isolates of *Bacillus* was recorded. These isolates, which did not form >0.5 cm diameter of inhibition zone, were not included in this study. The value of inhibition zone was converted into area of inhibition zone using the formula:

Area of circle = πr^2

Plant growth promoting attributes *in vitro*

The strains of *Bacillus* spp. which showed antagonistic ability to form >0.5 cm diameter of inhibition zone against *R. solanacearum* were screened for the expression of plant growth promoting attributes. The plant growth promoting attributes *viz*., phosphate solubilization, siderophore production and indole acetic acid (IAA) production possessed by bacteria were measured. Quantitative estimation of phosphate solubilization by *Bacillus* spp. was done as described by Mehta and Nautiyal (2001). Indole acetic acid produced by isolates of *Bacillus* was assayed colorimetric using ferric chloride-perchloric acid reagent (Vikram et al., 2007) and total soluble protein estimation was done according to Lowry et al. (1951). Siderophore production by *B. subtilis* isolates was measured using method described by Schwyn and Neilands (1987).

Assessment of antagonistic and plant growth promoting ability of *Bacillus* under glasshouse condition

Disease assessment

Among 47 isolates, 4 of *Bacillus* sp. *viz.,* DTBS-4, DTBS-5, JTBS-9 and METBS-31 were taken based on antagonistic and plant growth promoting attributes *in vitro* to evaluate their performance under glass house conditions in 2010-11. Aqueous suspensions (10^9 cfu/ml) of these strains *Bacillus* were prepared from 24 h old pure culture grown on TSA medium. Pots were filled with one kilogram of sterilized soil mixture containing vermiculite scaled, peat soil and sand in the ratio of 2:1:1 for raising the seedlings of tomato cultivars viz. Pusa Ruby (susceptible to bacterial wilt) and Arka Abha (resistant to bacterial wilt). 100 ml/per pot of 24 h old culture of each isolates of *Bacillus* was thoroughly mixed with the soil in the pot. After half an hour, 100 ml of 48 h old culture of *R. solanacearum* grown on CPG medium containing 2.5 x 10^9cfu/ml was mixed with the soil in each pot and five replications for each treatment was maintained. A set of treatment without adding antagonistic bacteria was maintained as control. Subsequently, 25 days old, five seedlings of tomato cv. Pusa Ruby and Arka Abha was transplanted in each pot separately. Wilt incidence was observed by counting the proportion of wilted plants in total plants per pot using 0-5 scale (Winstead and Kelman, 1952) and calculated wilt incidence/wilt intensity as mentioned by Schaad et al. (2001). Biological control efficacy (BCE) was calculated as described by Guo et al. (2004).

Assessment of plant growth parameters

The same procedure was followed as mentioned in disease assessment except pathogen *R. solanacearum,* which was not inoculated in the soil. After 45 days of inoculation, the effect of tested antagonist on growth was measured in terms of plant height (cm), fresh and dry weight (g) of root and shoot were assayed. Plants were measured for their height and uprooted whole plant with root from the pot. Root and shoot were cut from crown region, and fresh weight of root and shoot were recorded. The plant parts were dried at 60°C for three days and dry weight of root and shoot was recorded. The comparative growth promotion efficacy (GPE) by the strains of *Bacillus* was calculated as described by Singh et al. (2012):

$GPE = [(G_T-G_C)/G_C] \times 100$

Where, GPE is growth promotion efficacy, G_T is growth promotion by the treatment group and G_C is growth by the control.

Molecular characterization and genetic diversity

Extraction of genomic DNA

Total DNA of bacteria was extracted as described previously by using CTAB method (Murray and Thompson, 1980). The purified DNA was used for molecular characterization, detection of iturin production and 16s rRNA sequence and fingerprinting of *Bacillus* species.

***Detection of iturin D producing strains of Bacillus* spp.**

A set of primer (ITU-F: 5'- ATTGACGGAGGACGCGCCTAT-3' and ITU- R: 5'- CCGAGGA TCGCTACATCAAT-3') was developed from iturin D gene of *B. subtilis* with predicted product size of 617 bp. For validation of primer, 47 strains of *Bacillus* spp. including *B. cereus, B. licheniformis* and *B. subtilis* were performed in gradient thermal cycler (BIO RAD C100™Thermal cycler). The amplifications were carried out in a final volume of 20 µl of PCR master mix containing 4 µl of PCR buffer, 0.5 µl of dNTPs (Promega), 0.5 µl of each primer, 2.0 µl $MgCl_2$, 0.25 µl of Taq polymerase and 100 ng of DNA template. In each PCR experiment, a control without DNA template was used as negative control. The PCR reactions were run with initial denaturation of 5 min at 94°C, 30 cycles each consisting of 94°C for 30 s, 62°C for 45 s and 72°C for 1 min and final extension 72°C for 5 min. A 15 µl aliquot of each amplified PCR product was separated on a 1.0% agarose gel in 0.5% TBE buffer, gel electrophoresis unit was run on 70 V for 1.0 h and stained with ethidium bromide and visualized under gel documentation (BIORAD, GEL DOC™ XR+ with image Lab™software).

PCR amplification of 16S rRNA and sequencing

For identification of 44 strains of *Bacillus* spp. (Table 1), a PCR technique was used as described by Wattiau et al. (2001). For sequence analysis of 21 strains of *Bacillus* spp. (Table 1), another set of universal primer (UNI_OL5 :5`-GTGTAGCGGTGAAATGCG-3`) UNI_OR (5` ACGGGCGGTGTGTACAA-3`) based on 16s rRNA for bacteria was used as described by Sauer et al. (2005). 15 µl of PCR product were analyzed in 1.5% agrose gel at 80 V for 1.0 h, and the gel was viewed under gel documentation system (BioRad,GEL DOC™ XR+ with image Lab™software) and photographed. Sequencing of 21 strains of *Bacillus* species belonging to different agro-climatic region was performed using ABI3730XL sequencer and reactions were analyzed on a capillary sequencer. 16S rRNA sequences of all the strains were compared with available database with high sequence matching >95% using GenBank BLAST N to determine approximate (≈700 bp) phylogenetic affiliation. The phylogenetic relationships were inferred by MEGA4 (Tamura et al., 2004, 2007). The sequence data were submitted in NCBI and accession no. of each strain of *Bacillus* spp. is given in bracket as follows, DTBS-4 (JQ688021), DTBS-5 (JQ688022), DTBS-6 (JQ688023), HPTBS-4 (JQ688024), HPTBS-5 (JQ688025), JHTBS-2 (JQ688026), JHTBS-6 (JQ688027), JHTBS-

Table 2. Effect of antagonistic isolate of *Bacillus* sp. on wilt incidence on susceptible and resistant cultivars of tomato and biocontrol efficacy cultivars under glasshouse condition.

Isolate of *Bacillus* sp.	Pusa Rubi(susceptible to bacterial wilt)		Arkha Abha(resistant to bacterial wilt)	
	Wilt incidence (%)	Biocontrol efficacy (%)	Wilt incidence (%)	Biocontrol efficacy (%)
HTBS-4	21.2^{c}	66.7	11.5^{c}	47.7
DTBS-5	14.3^{d}	77.6	7.6^{d}	65.9
JTBS-9	27.3^{bc}	57.1	15.7^{b}	19.5
METBS-31	29.4^{b}	53.9	19.9^{b}	23.2
Control	63.3^{a}	-	22.0^{a}	-

Means followed by the same letter within a column are not significantly different as determined by LSD test (α = 0.05). Data present means of the experiment with 5 replications each.

7 (JQ688028), JHTBS-8 (JQ688029) JHTBS-9 (JQ68830), JHTBS-21 (JQ68831), JTBE-23 (JQ68832), JTBS-9 (JQ68833), JTBS-17 (JQ68834), METBE-35 (JQ68835), METBS-32 (JQ68836), UTTBS-1 (JQ68838) and UTTBS-3 (JQ68839).

Genotypic diversity

The genomic DNA (50 ng) from 47 strains of *Bacillus* spp. including *B. cereus, B. subtilis* and *B. licheniformis* used to perform template in 25 µl of reaction mixture for fingerprinting was carried out using BOX primer (BOXA1R 5'- CTACGGCAAGGCGACGCTGACG-3') described by Schaad et al. (2001). The PCR product of 15 µl were separated by electrophoresis on a 1.5% agarose gel in 0.5 x TAE buffer for 6.5 h at 100 V. Gel was stained with ethidium bromide and photographed on gel documentation system. For cluster analysis, the data were converted to a binary matrix, where the digit 1 or 0 represented the presence of or absence of DNA band in the gel. NTSYS (2.02e version) software was used to analyze the fingerprinting. The similarity coefficient of BOX-PCR fingerprint was calculated with Pearson coefficient. Cluster analysis of the similarity matrix was performed by unweighted pair group method using arithmetic averages (UPGMA) algorithm (Saitou and Nei, 1987).

Data analysis

The analysis of variance for antagonistic ability, PGP attributes was performed by using standard procedure (Gomez and Zomez, 1984). Mean comparisons were conducted using a least significant difference (LSD) test (P = 0.05).

RESULTS

Antagonistic property of *Bacillus* isolates

Out of 250 isolates of *Bacillus* screened during preliminary test, 44 isolates collected from different agro-climatic regions of India and *B. subtilis, B. cereus* obtained from IMT, Chandigarh and *B. licheniformis* from Division of Plant Pathology, IARI, New Delhi presented inhibition activity against *R. solanacearum*. Hence, these 44 bacterial isolates were considered as bacteria with antagonistic characteristics. Maximum growth of *R. solanacearum* was inhibited by strain DTBS-5, which formed area of inhibition zone of 5.5 cm^2 followed by METBS- 31 (3.6 cm^2) and METBE- 35 (3.4 cm^2) *in vitro.* Whereas the other strains of *Bacillus* species had a wide range of antagonistic activity to form inhibition zone ranging from 0.51 to 2.94 cm^2 in laboratory *in vitro* test (Table 1). Significant variation in formation of inhibition zone against *R. solanacearum* was recorded.

Antagonistic strains of *Bacillus* showed significant differences for their abilities to suppress tomato bacterial wilt. Out of 47 strains showing potential for antagonistic ability, 4 strains namely HTBS-4, DTBS-5, JTBS-9 and METBS-31 were chosen for antagonistic and plant growth promoting ability under greenhouse condition. Strain DTBS- 5 showed the lowest value of wilt incidence of 14.3 and 7.6% in Pusa Ruby and Arka Abha cultivars, which was the highest biocontrol efficacy (77.6 and 65.9%) against *R. solanacearum*, respectively (Table 2). While other strains HTBS-4, JTBS-9 and METBS-31 also reduced the disease incidence as significantly compared to control. However, Arka Abha showed resistance against the disease under glass house condition, and further reduced wilt incidence and improved further biocontrol efficacy of *Bacillus* strains.

Plant growth promoting attributes

Screening of soil and endophytic bacteria for plant growth promoting activities were studied. The strains having antagonistic property >0.5 mm diameter of inhibition zone was considered for plant growth promoting attributes. 44 isolates of *Bacillus* were tested for solubilizing phosphorous *in vitro* and all strains have ability to solubilize the phosphorus (Table 1). However, strain JTBS-9 solubilize the highest phosphorus (72.7 µg/ml) followed by HPTBS-4 (69.0 µg/ml) (Table 1). A significant variation in solubilizing phosphorous was recorded among the strains of *Bacillus* spp. The bacteria also had ability to produce siderophore, which is an iron chelating agent. Strains DTBE-9 and UTTBS-3 produced maximum (1.30 µg/ml) siderophore in the medium followed by HPTBS-4 (1.17 µg/ ml) *in vitro.* Besides these strains, other strains of *Bacillus* produced siderophore ranging from 0.57 to 1.13 µg/ml in the medium. Indole acetic acid production is one of the criteria for screening of PGP bacteria and all

Table 3. Effect of antagonistic isolate of *Bacillus* sp. on growth and biomass of susceptible and resistant cultivars of tomato cultivars under glasshouse condition.

Isolate of *Bacillus* sp.	PusaRubi							ArkaAbha						
	Shoot length (cm)	Fresh shoot wt. (g)	Dry shoot wt. (g)	Root length (cm)	Fresh Root wt. (g)	Dry root wt. (g)	GPE (%)	Shoot length (cm)	Fresh shoot wt. (g)	Dry shoot wt. (g)	Root length (cm)	Fresh root wt. (g)	Dry root wt. (g)	GPE (%)
HTBS-4	46.6^{a}	39.4^{a}	5.3^{ab}	6.9^{a}	5.2^{ab}	1.1^{a}	14.3	4.8^{ab}	43.1^{bc}	5.2^{ab}	10.1^{ab}	7.7^{a}	0.9^{a}	7.0
DTBS-5	41.0^{a}	36.7^{a}	5.0^{ab}	6.2^{a}	5.7^{ab}	1.2^{a}	10.7	3.5^{ab}	43.4^{abc}	5.6^{ab}	9.3^{b}	7.6^{a}	1.0^{a}	15.7
JTBS-9	47.9^{a}	42.0^{a}	6.1^{a}	6.9^{a}	6.3^{a}	1.3^{a}	32.1	56.0^{a}	49.0^{ab}	6.6^{a}	11.8^{a}	8.0^{a}	1.1^{a}	35.1
METBS-31	46.3^{a}	37.4^{a}	5.0^{ab}	5.9^{a}	5.5^{ab}	1.1^{a}	8.9	57.1^{a}	48.2^{a}	6.2^{ab}	9.7^{b}	7.9^{a}	0.9^{a}	21.1
Control	41.2^{a}	31.0^{b}	4.7^{b}	6.0^{a}	5.1^{b}	0.9^{a}	-	47.1^{b}	39.8^{c}	4.8^{b}	8.5^{b}	5.9^{b}	0.9^{a}	-

wt.: Weight; Means followed by the same letter within a column are not significantly different as determined by LSD test (α = 0.05). Data present means of the experiment with 5 replications each.

all tested strains of *Bacillus* spp. produced IAA ranging from 41.0 to 288.5 µg/ml. However, UTBS- 33 produced the highest amount of IAA 288.5 µg/ml followed by UTBS-33 (254.3 µg/ml) *in vitro*.

Out of 47 strains of *Bacillus* spp. 4 strains viz. HTBS-4, DTBS-5 JTBS-9 and METBS-31 were used to study the effect of these bioagents on plant height and biomass production under glass house conditions. Plants treated with strain JTBS-9 of *Bacillus* sp. had maximum fresh shoot weight of 42.0 and 49.0 g and dry weight of 6.1 and 6.6 g of Pusa Ruby and Arkha Abha after 45 days of transplanting, respectively, which had high significant differences among other bioagents HTBS-4, DTBS-5 and METBS-31 and untreated control (Table 3). Fresh weight and dry weight of root and shoot of the cultivars treated with the strain of *Bacillus* was significantly increased and found variable in treated and untreated plants.

Morever, plant growth efficiency was found higher in JTBS-9 treated Pusa Ruby and Arka Abha, 32. 1 and 35.1% based on dry weight of root and shoot of the plant. Although, the highest dry weight of root of both cultivars was found in treated with JTBS-9 but variation in dry weight of root treated with bioagents and untreated control was insignificant.

Characterization of bacteria

The cells of *Bacillus* isolates were rod shaped, some cells are in pair, Gram positive and endospore forming. The 250 strains of *Bacillus* spp. were analyzed based on morphological observations, physiological and biochemical characters, showing their similarities to *Bacillus* species like endospore formation of the cell, swelling of bacillary body and no growth in glucose broth under anaerobic condition.

The *Bacillus* hydrolyzed the starch and gelatin and make clear zone. They also showed oxidase test, growth in 7% NaCl, and citrate utilization tested positive. The *Bacillus* strains showed similar properties in utilization of carbon sources and biochemical reactions as reported earlier for *Bacillus* spp.

Molecular characterization of *Bacillus* spp.

Based on the large multiple alignments of *Bacillus* 16S rRNA sequences, the two PCR primers, *Bsub*-5F and Bsub-3R were chosen (Wattiau et al., 2001), which was specific to *B. subtilis* group as reported and predicted to specifically amplify at 595 bp DNA fragments of *B. subtilis.* All strains of *Bacillus* spp. were detected as positive with this test except *B. cereus*, which were very close from a taxonomic point of view (Table 1).

21 strains of *Bacillus* species were confirmed to belong to the *B. subtilis* group which included *B. licheniformis*, *B. subtilis*, *Bacillus pumilus*, *B. atrophaeus*, and *Bacillus amyloliquefaciens* by using 16S rRNA based primer. However, these strains were isolated from rhizospheric soil and endophytic of tomato plants from different agro-climatic regions belonging to *B. subtilis* group.

Iturin production

A primer was developed to screen iturin producing isolates of *Bacillus* spp. isolated from diverse climatic conditions across India. PCR amplification for iturin locus showed 617 bp amplification in 11 strains out of 47 strains of *Bacillus* spp. Including *B. subtilis*, *B. cereus* and *B. licheniformis* (Figure 1).

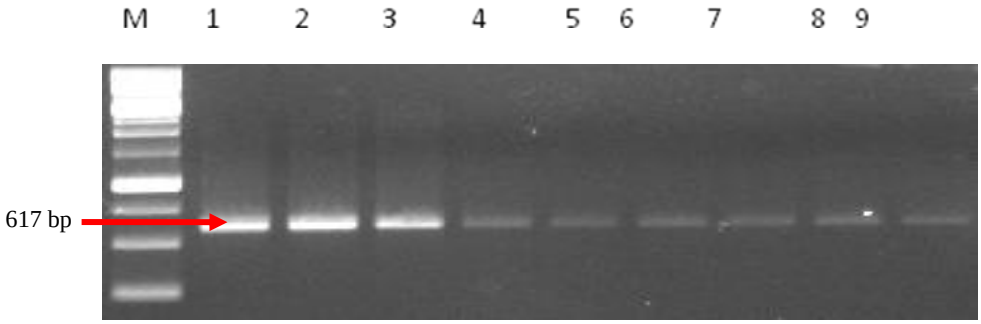

Figure 1. The amplified DNA fragments at 617 bp produced strains of *Bacillus* spp. by ITU primer isolated from rhizosphere and endophyte of tomato plants. Lane M: 1 Kb DNA Ladder, lanes 1: *Bacillus subtilis*, 2: UTTBS-1, 3: JHTBS-2, 4: UTTBS-3, 5: HPTBS-4, 6: JHTBS-5, 7: DTBS-4, 8: METBS-32, 9: METBE-35.

All the strains producing iturin were soil inhabitant under diverse climatic conditions belonging states Delhi, Uttarakhand, Himachal Pradesh, Jharkhand and Meghalaya of India.

Genomic fingerprinting

The fingerprinting patterns of 47 strains of *Bacillus* spp. isolated from rhizosphere and endophytic of tomato plants from different agro-climatic regions of India was generated by BOX- PCR considered as 5 - 15 fragments ranging in size of about from 200 to 4.5 kb (Figure 2b). The variations in number of fragments, their size among the strains of *Bacillus* spp. were observed. The highest 15 fragments were noted in strain MTBE-35. Computer assisted analysis of the BOX-PCR fingerprinting showed that all tested strains could be grouped into five distinct clusters at 50% similarity coefficient. Cluster 1 represented the 40 strains of *B. subtilis* and unidentified *Bacillus* spp., cluster 2 comprised three strains including *B. cereus,* JHTBS-6 andJ HTBS-8. Whereas strain DTBE-4 and UTBS-24 clustered in cluster 3. *Bacillus licheniformis* and JTBS-17 formed clusters 4 and 5, respectively. Cluster 1 further separated into 7 subgroups A, B, C, D, E, F and G at >75% similarity coefficient (Figure 2a) and subgroup A contained the highest 18 strains of *Bacillus* spp. irrespective of agro-climatic regions belonging to state Manipur, Uttar Pradesh, Delhi, Jammu and Kashmir followed by subgroup C comprising of 9 strains *viz.*, JHTBS-5, JHTBS-7, METBS-32, UTBE-25, UTBE-29, UTBS-30, UTBE-31, METBS-31and UTBE-30. Moreover, sub group B, D, E, F and G comprised 4, 4, 2, 1 and 2 numbers of strains of *Bacillus* sp., respectively.

Genetic diversity of 21 strains of *Bacillus* spp. based on the partial 16S rRNA sequence analysis (≈ 700 bp) isolated from endophytic and rhizospheric of tomato plant were determined with homology of 95-100. These strains of data base were grouped along with sequences of other *Bacillus* spp. obtained from NCBI clustered. Based on the grouping, strain JHTBS- 8, JHTBS-9, JHTBS-2, JTBE-23 were very close to *B. subtilis*, strain JHTBS-1 close to *B. amyloliqueficience*, whereas JHTBS-6 and JTBS-9 were very close to *B. cereus* K17 and HTBS-4 close to *Bacillus* sp. YXA. However, the remaining 15 isolates made separate 3 groups (Figure 3). The grouping of the strains was not based on climatic conditions and also not disguised by their nature of inhabitants.

DISCUSSION

Bacterial wilt of tomato is a serious disease in India and severely infected field as high as 95% disease incidence was caused by *R. solanacearum* (Mishra et al., 1995; Singh et al., 2012). Managing the bacterial disease by biocontrol agents has become increasingly important. Among various bioagents, rhizobacteria and endophytic bacteria have an impressive effect which protect the soil borne bacterial pathogens as well as improve plant growth (Glick et al., 2007; Almoneafy et al., 2012; Singh et al., 2012).

In our study, rhizobacteria and endophytes belonging to genus *Bacillus* were isolated from tomato plants from different agro-climatic conditions to find the potential strains representing high bacterial wilt control and also to offer several plant growth promoting attributes such as phosphorus solubilizing, siderophore production and indole acetic acid production to improve health and growth of tomato plant. *In vitro* study, 250 isolates of *Bacillus* spp. were tested and those that formed >0.5 cm diameter of inhibition zone were considered for antagonistic ability, in which 47 strains represented remarkable antagonistic activity against *R. solanacearum*. The strains DTBS-5, METBS- 31, METBE- 35, UTBE- 26 and UTBE-30 have potential for highest inhibition effect against bacterial pathogen. These results confirm the previous studies reporting the antagonizing activity of *Bacillus* species against *R. solanacerarum* (Li et al., 2008; Almoneafy et al., 2012;

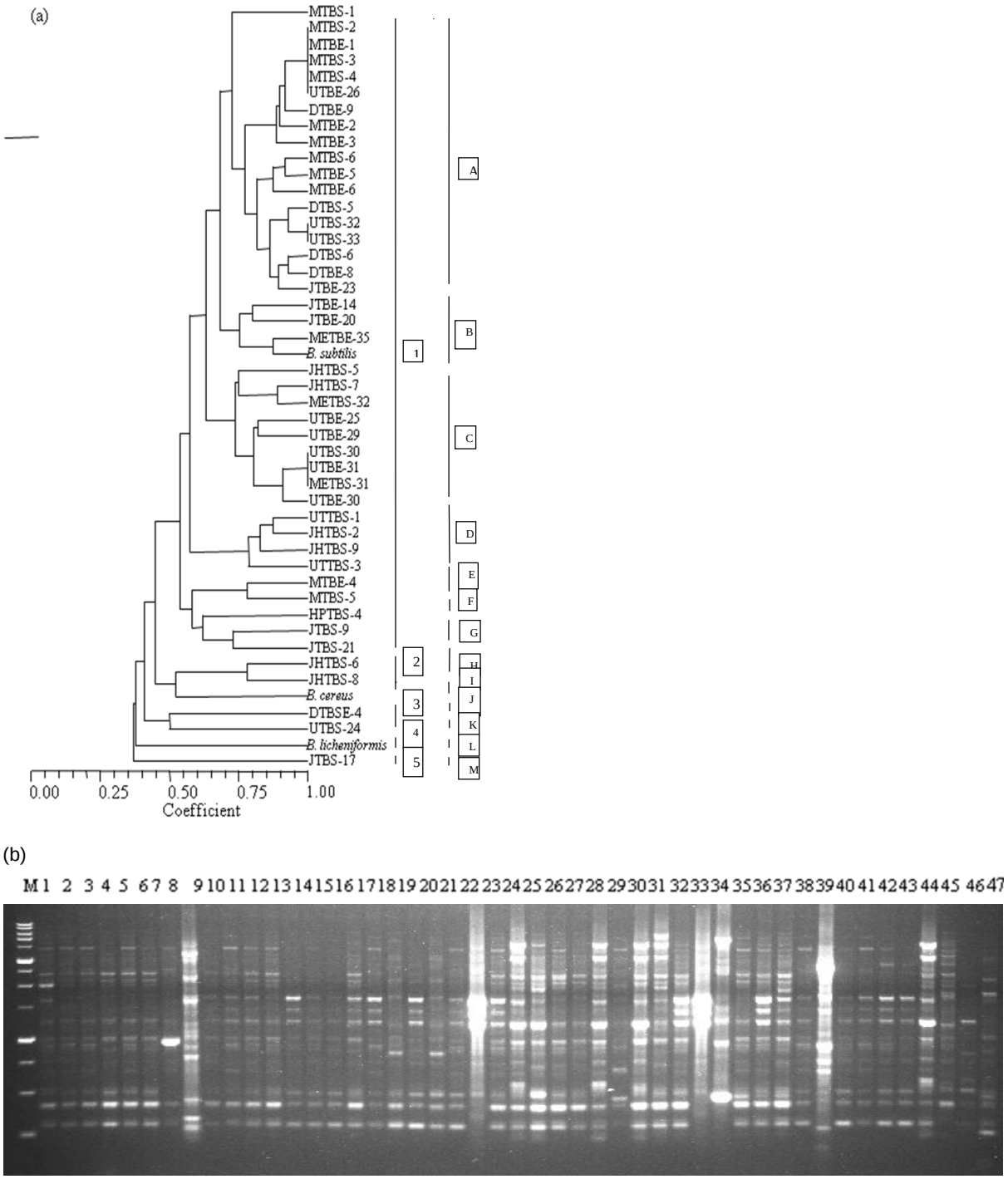

Figure 2. (a) Cluster analysis of genetic diversity of 47 strains of *Bacillus* spp. reaction by using BOX-PCR. The phylogenic tree was constructed by the UPGMA method for the similarity matrix generated using Pearson's correlation coefficient applied to the whole patterns. The minimum similarity coefficient of all isolates was used to define distinct groups, which are labeled numerically. Distinct subgroup of genomic variability of Bacillus spp. isolates are labeled alphabetically A - M. (b)The amplified DNA fragments produced by BOX primer for strains of *Bacillus* species. Lane name with respective strains. Lane M: 1 Kb DNA ladder, Lanes 1 - 12: Strains from Manipur (MTBS-1, 2: MTBS-2, 3: MTBE-1,4: MTBE-2, 5: MTBS-3, 6: MTBS-4, 7: MTBE-3, 8: MTBE-4, 9: MTBS-5, 10: MT*BS*- 6,11: MT*BE-5,* 11: MTBS-6, 12: MTBE-5), lanes 13 and 15: strains from Uttarakhand (UTTBS-1 and UTTBS-3) lanes 14, 17 - 21: strains from Jharkhand (JHTBS-2, JHTBS-5, JHTBS-6, JHTBS-7, JHTBS-8 and JHTBS-9), lane 16: strain from Himachal Pradesh (HPTBS-4), lanes 22 - 26: strains from Delhi (DTBSE-4, DTBS-5, DTBS-6, DTBE-8, DTBE-9), lanes 27 - 32): strains from Jammu and Kashmir (JTBS-9, JTBE-14, JTBS-17, JTBE-20, JTBS-21 and JTBE-23), lanes 33- 41: strains from Uttar Pradesh (UTBS-24, UTBE-25, UTBE-26, UTBS-32, UTBS-33, UTBE-29, UTBS-30, UTBE-30, UTBE-31, lanes 42- 44: strains from Meghalaya (METBS-31, 43: METBS-32, 44: METBE-35), 45: *B. subtilis*, 46: *B. licheniformis*, 47: *B. cereus.*

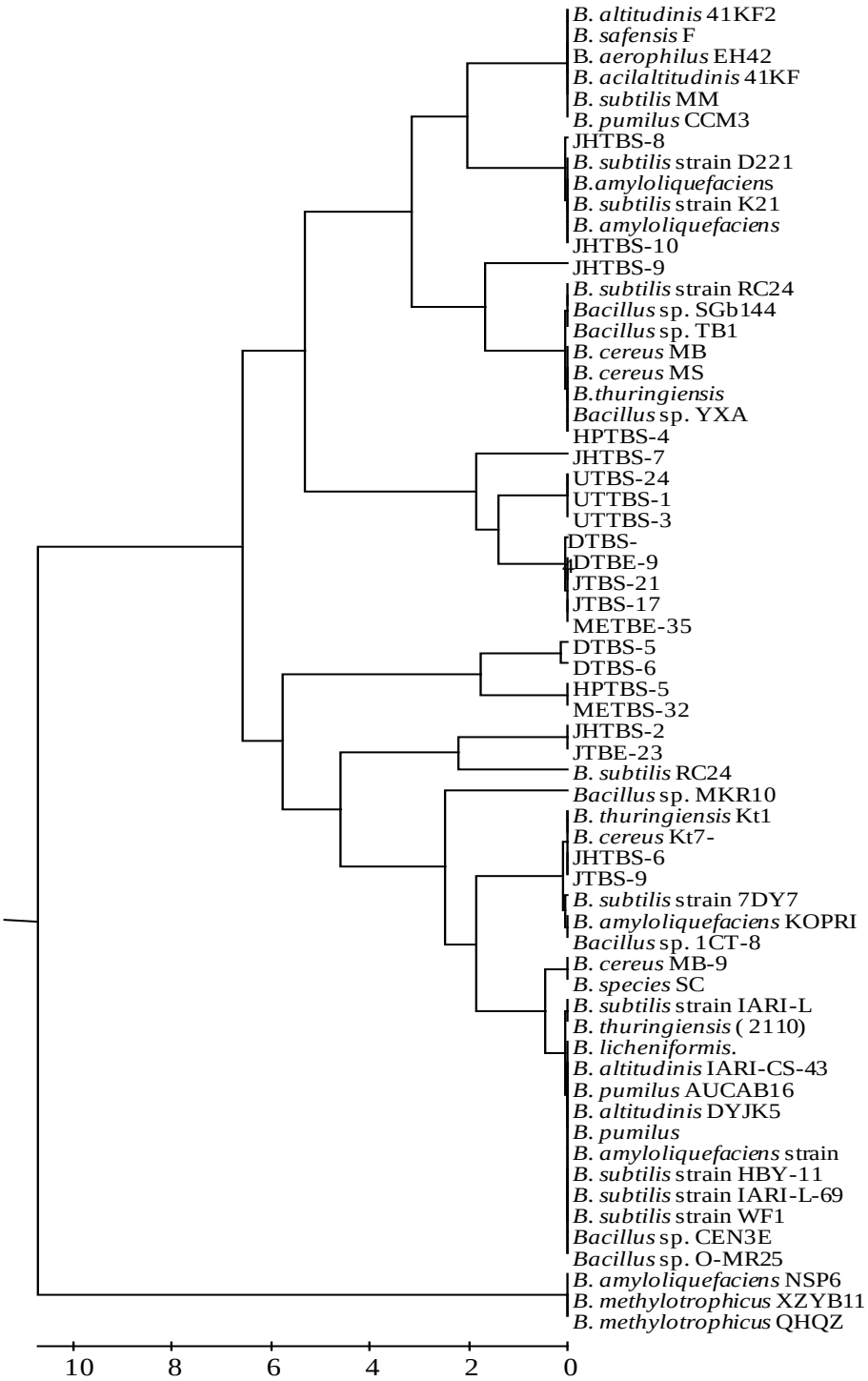

Figure 3. Phylogenetic analysis based on 16S r RNA sequences of 21 isolates of *Bacillus* spp. showing the position with respect to related species available from NCBI database. The evolutionary history was inferred using the UPGMA method. There was a total of 650 positions in the final dataset and phylogenetic analyses were conducted in MEGA4.

Singh et al., 2012). Growth inhibition zones on the agar media may be formed due to chemicals like antibiotic substances with broad spectrum of activity or more specific bacteriocin (Gross and Vidaver, 1990) or production of siderophore or both of them produced by bacteria (Adesina et al., 2007; Lemessa and Zeller, 2007).

In this study, CPG medium was used for *in vitro* screening assay, because the medium was suitable for *R. solanacearum* growth and agrees with other earlier studies, which reported that the type of culture medium strongly affects antagonistic activity mediating the production of chemical substances responsible for inhibition (Montesinos et al., 1996). *Bacillus* species are known as very efficient producers of antibiotic molecules of three main family, surfactins, iturins and fengycins. *B. subtilis* has the potential to produce > 24 structurally diverse antimicrobial compounds (Stein, 2005), which have been reported for their inhibitory activity against plant pathogens.

Variability in production of PGP attributes were found in *Bacillus* spp., which are influenced by various factors such as nutritional richness, physiological and growing status of the bacterium (Upadhyay et al., 2009). Strains JTBS-9, HPTBS-4, MTBS-5 and MTBS-6 have demonstrated the high ability to solubilize inorganic phosphate *in vitro*. *Bacillus* spp. is capable of increasing availability of phosphorus in soil. Phosphorus solubilizing bacteria has also been isolated from soil (Mehta and Nautiyal, 2001) and endophyte (Rajendran et al., 2012), which supported our findings. Siderophore production is another plant growth promoting attributes, which is produced by rhizobacteria and endophytes (Beneduzi et al., 2008). Strains UTTBS-3, HPTBS-4 and JHTBS-5 produce >1.0 µg/ml siderophores *in vitro*. In our study, all the strains of *Bacillus* spp. produced IAA, which range from 41.0 - 288.5 µg/ml. However, strains UTBS-33, JHTBS-5 and JTBE-20 have high potential to produce IAA, which help in promoting plant growth (Loper and Schroth, 1986). It has been observed that strain like DTBS-5 has the highest antagonistic ability to suppress the bacterial wilt incidence but has lower IAA production. It might be due to inactivation of gene responsible for IAA biosynthesis which causes reduction in IAA concentration, resulting in low plant growth promotion activity (Idris et al., 2007). The overall result showed that strains isolate HPTBS-4, JHTBS-5, UTTBS-3 and UTBS-33 have better PGP attributes, which may be good candidates for PGP trait. Moreover, the strains UTTBS-3 and JHTBS-5 produce iturin antibiotic, which was confirmed through molecular techniques.

Molecular characterization the 47 strains of *Bacillus* spp. was performed for further confirmation. The primer based on 16s rRNA gene specific to *B. subtilis* group was applied for characterization of isolate in which *B. licheniformis*, *B. subtilis*, *B. pumilus*, *B. atrophaeus*, and *B. amyloliquefaciens* are included (Wattiau et al., 2001). All the strains used in this study were amplified except *B. cereus*. It indicates that these strains isolated from rhizospheric soil and endophyte of tomato plants from different agro-climatic regions belong to *B. subtilis* group as earlier reported by Wattiauet al. (2001). But in contrast to the earlier study, we found that the strains JHTBS-6 and JTBS-8 are grouped with *B. cereus* in BOX- PCR and and JHTBS-6 in 16S rRNA sequence analysis which are amplified.

The dendrogram (Figure 2a) was generated by use of the Jacord coefficient to analyze BOX-PCR data. Our result discriminated *B. cereus*, *B. subtilis* and *B. licheniformis* along with other strains of *Bacillus* spp. In contrast to our result, it did not reliably discriminate between *B. cereus* and *B. thuringiensis* (Kim et al., 2002). Another genetic diversity study of *Geobacillus* and *Bacillus* strains isolated from a volcanic region compared different molecular techniques such as *rpo*B sequence analysis, repetitive extragenic palindromic-PCR (REP-PCR) and BOX-PCR (22). Upadhyay et al. (2009) reported that *Bacillus* and *Bacillus* derived genera dominated in rhizoplane of soil of wheat and found a lot of genetic diversity in *Bacillus*. Genetic variation in strains of *Bacillus* spp. may be due to mutation and other genetic changes like recombination. Though BOX-PCR allowed grouping of strains but it was not possible to clearly distinguish the iturin antibiotic producing strains from other non-iturin producing strains. In our case, since we took only those strains of *Bacillus* having antagonistic ability and plant growth promoting attributes, hence they could not be differentiated genetically. Moreover, the results of BOX-PCR did not generate any molecular marker that was associated with antagonistic and plant growth activity.

Conclusion

The strains of *Bacillus* spp. isolated from rhizospheric soil and endophyte of tomato posses both tracks, that is, antagonistic and PGP attributes and few strains are able to produce iturin antibiotics. However, non iturin producing strains have better antagonistic property, which indicates that they produced some other antibiotics. *B. subtilis* strains were discriminated by using 16S rRNA primer and BOX-PCR. The BOX-PCR genomic fingerprinting could be used as highly discriminatory technique to determine the genetic relatedness and diversity among the strains of *Bacillus* species.

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Full Length Research Paper

Comparative efficacy of Internalin C-based peptide and listeriolysin O-based enzyme linked immunosorbent assays for serodiagnosis of listeric infection in goats

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This is the first study ever carried out to develop and evaluate internalin C (InlC)-based serological assay for diagnosis of *Listeria monocytogenes* (LM) infection in goats using synthetic peptide as an antigen. Nine peptides representing major antigenic domains of InlC, a novel protein linked to the virulence of LM, were identified, analyzed, synthesized and employed in indirect enzyme-linked immunosorbent assay (ELISA) to evaluate their diagnostic potential using sera of goats experimentally inoculated with live and killed LM and from apparently healthy goats. Sera were screened by standardized indirect ELISA to reveal the antibodies against InlC (AInlC) as well as listeriolysin O (ALLO). Overall, the result revealed that the AInlC titres are lower than the ALLO titres. However, a fair correlation was observed between the titres of AInlC and ALLO in experimentally infected as well as apparently healthy goats. Based on the results obtained by both the ELISAs, it is suggested that InlC peptides alone may not serve as a suitable diagnostic antigen in indirect based ELISA for serodiagnosis of listeric infections. Further, there is need for identification, synthesis and evaluation of appropriate synthetic peptide(s) for essential virulence markers of listeriae whether existing or new marker, all need to be explored for developing an ultimate sensitive and specific rapid sero-diagnostics marker against listeriosis.

Key words: *Listeria monocytogenes,* Internalin C, Listeriolysin O, Peptide and enzyme-linked immunosorbent assay (ELISA).

INTRODUCTION

Listeriosis is a bacterial disease characterized by neural, visceral and reproductive clinical entities, usually manifested as septicaemia, meningitis, encephalitis and abortion in humans and animals. It is mainly transmitted by ingestion of contaminated food and the disease is particularly common in ruminants fed on silage (Wagner and McLauchlin, 2008). It has emerged as an important food borne disease in human beings, especially in the developed world. Out of the eight known species of the *Listeria*, including the two new species *Listeria marthii* (Graves et al., 2010) and *Listeria rocourtiae* (Leclercq et al., 2010); *Listeria monocytogenes* and *Listeria ivanovii* are the only two pathogenic strains responsible for the illness. *L. monocytogenes* is responsible for 85% of animal cases and about 98% of human cases (Liu, 2006) with a very high mortality rate (20 to 30%) and ability to cause severe disease among the pregnant women, neonates and immune-compromised individuals (Esteban et al., 2009).

Many serological and molecular tests have been attempted

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since the discovery of the bacterium with the aim of identifying the bacteria quickly, and with a greater degree of sensitivity (Jadhav et al., 2012; Shoukat et al., 2013). The humoral immune response is normally detected during listerial infection even in the absence of clinical symptoms. Many serodiagnostic assays employing the somatic (O), flagellar (H), cold-extracted and sonicated antigens of *Listeria* spp. have been developed for screening the animal and human listeriosis cases. However, these conventional assays cannot be relied upon for their poor specificity and sensitivity (Berche et al., 1990). Outer membrane protein (OMP) of *Listeria* spp. has also been used for developing Genus-specific as well as *L. monocytogenes*-specific enzyme-linked immunosorbent assays (ELISA) (Chen and Chang, 1996). However, these assays fail to discriminate between pathogenic and non-pathogenic *Listeria* strains. A number of virulence markers of *Listeria* species capable of eliciting the antibody response during listerial infection such as listeriolysin-O (LLO) (Berche et al.,1990); internalins (InlA, InlB, InlC, InlC2, InlJ etc.), the leucine rich repeat (LRR) proteins of *Listeria* spp. produced by virulence-linked family of genes (Boerlin et al.,2003); Act A protein associated with cell-to-cell spread of the agent (Ellin Doyle, 2001), two phospholipases C namely the PI-PLC encoded by *plc*A gene and the PC-PLC encoded by *plc*B (Chaudhari et al., 2004a,b); and the autolysin p60 protein (Hess et al., 1996). Out of the mentioned markers, LLO a 58-kDa secreted protein is one of the most important virulence factor produced by *L. monocytogenes* which can be detected soon after clinical onset of listeriosis and antibodies persist for at least several months (Berche et al., 1990; Shoukat et al., 2013). LLO based ELISA had been used (Low and Donachie, 1991; Low et al., 1992; Boerlin, 2003) however, LLO cross reacts with streptolysin, perfrinolysisn, suilysin and hence need prior adsorption at least with streptolysin O (SLO) before performing LLO-based ELISA (Berche et al., 1990). Hence, other virulence marker are being searched extensively to remove this pothole and in this regard, Internalin group of protein had proved potential especially InlC - being an immunodominant and virulence-linked protein, had shown promising result as diagnostic antigen in ELISA for screening the human clinical cases (Grenningloh et al., 1997) and experimental listeric infection in ewes (Zundel et al., 2007), where it proved superior to LLO-based ELISA for reliable diagnosis of listeric infection. It is noteworthy that barring the two non-pathogenic serovars of *L .monocytogenes* namely 4a and 4c (Liu et al., 2007), the InlC has been reported to be secreted by the pathogenic strains of all the serovars of *L. monocytogenes* including the most important serovars; that is, ½ a, ½ b and 4b; as well as the only serovar of *L. ivanovii*; that is, 5. Therefore, it would be rational to study the diagnostic potential of this important and novel virulence marker as a diagnostic antigen in a serological assay for reliable diagnosis of listeric infection by the pathogenic species of Listeria in man and domestic animals, particularly the goats, which are one of the most susceptible species. In view of the above facts, the present study was undertaken to develop and evaluate an ELISA employing synthetic peptides of InlC, for rapid and reliable serodiagnosis of listeric infection using sera of goats experimentally infected with live and killed LM as well as serum samples collected of apparently healthy goats. The developed InlC based indirect ELISA was compared further with LLO based indirect ELISA using the same sera samples to reveal it diagnostic sensitivity and specificity.

MATERIALS AND METHODS

Reference strains

The standard strain of *L. monocytogenes* 4b (MTCC 1143) was procured from IMTECH, Chandigarh. The strain was tested for its purity by morphological and biochemical characterization. The pathogenic potential was tested by *in vivo* pathogenicity tests in mice and the strain was passaged twice in mice for revival of its pathogenicity. The strain was maintained in the laboratory by monthly sub-culturing in brain heart infusion (BHI) broth.

Animals

The animal experiments were carried out after approval from Institutional Animal Ethics Committee. In brief eight healthy male Black Bengal goats (12 to 18 months old) were procured from the Livestock Production Research Section, Indian Veterinary Research Institute, Izatnagar, India. Prior to inclusion of these animals in the study, absence of listeric infection was ascertained by blood culturing as well as testing the serum of animal by Indirect based LLO ELISA. During the entire course of the experiment, the animals were fed *ad libitum* with fodder, concentrate and water. The goats were divided into three major groups, having five goats in group I, one goat in group II and two goats in group III, respectively. All the five goats of group I were orally infected each with 3×10^9 live cells of pathogenic *L. monocytogenes* MTCC, while the goat in group II was subcutaneously inoculated with 3×10^9 killed cells of pathogenic *L. monocytogenes* MTCC 1143. The two goats of group III, were kept as control of which one was given oral sterile PBS, whereas, another goat was subcutaneously inoculated with sterile PBS. The animals were kept under routine clinical observation and their rectal temperatures were recorded daily for 14 days post infection (PI). Besides, the presence of *L. monocytogenes* was determined in samples of blood and swabs from the rectum and nasal cavity, which were collected from all the experimental (infected and non-infected) animals till the end of the study; that is, day 60 PI. The bacteriological samples were processed immediately to isolate the pathogen whereas serum samples were stored at -20°C until tested by ELISA. Use of experimental animals complied with the guidelines of the Committee for the Purpose and Supervision of Experiments on Animals (The Gazette of India).

Indirect LLO enzyme-linked immunosorbent assay (ELISA)

Indirect based LLO ELISA was performed using purified LLO available at Division of Veterinary Public Health, Indian Veterinary Research Institute, Izatnagar. The purity of LLO was checked with SDS-PAGE while protein concentration was estimated using Bradford assay. Cross reactivity of LLO with SLO was avoided by

Table 1. Peptide sequences for Internalin C.

Code of candidate peptide	Sequence of peptide	Orientation	Position of amino acid in InlC sequence	Length
InlC.1M	TGSGTKVQAESIQRPTPINQ	MAP	25 - 44	20
InlC.2M	GVQNFNGDNSNIKSLA	MAP	75 - 90	16
InlC.3L	ELSMNRNRLKNLNGIPSAC	Linear	122 - 140	19
InlC.4L	VDNNELRDTDSL	Linear	146 - 157	12
InlC.5L	LSIRNNKLKSIVM	Linear	166 - 178	13
InlC.6L	HGNEITNTGGLTRLKKVNWI	Linear	191 - 210	20
InlC.7L	DLTGQKCVNEPVRYQPE	Linear	211 - 227	17
InlC.8L	ELYITNTVKDPDGRWIS	Linear	227 - 243	17
InlC.9L	DLTGQKCVNEPVRYQPELYI TNTVKDPDGRWI	Linear	211 - 242	32

adsorption of goat sera with SLO as per the method described by Berche et al. (1990) with certain modifications. These SLO-adsorbed goat sera were then used in the indirect plate ELISA to screen for ALLO as per the method described by Low et al. (1992).

Prediction and synthesis of InlC peptides

The protein sequences of *L. monocytogenes* and *L. ivanovii* for Internalin C gene were obtained from NCBI database. Prediction of peptides corresponding to antigenic epitopes of InlC was performed using bioinformatics software such as Protean-DNASTAR followed by basic local alignment search tool (BLAST) analysis of all individual peptides to confirm their individual specificity. In brief, analysis of the sequences of InlC protein, by Megalign revealed more than 91% homology between *L. monocytogenes* and *L. ivanovii*. The BLAST analysis proved conserved nature of this protein in both the pathogenic species. Nine short peptides (12-32 amino acids) were tentatively selected as 'probable candidate peptides' based on their hydrophobicity, antigenecity and surface probability with turn regions in 'Protein antigenecity plot' performed by 'PROTEAN' program of DNA-STAR vis-à-vis high score of these nine peptides obtained by on-line analysis from National Institute of Health (NIH), USA. The top scores of BLAST analysis of these nine 'probable candidate' peptides showed very high specificity for InlC of *L. monocytogenes* (~100 to 66%) and of *L. ivanovii* (~100%), and therefore, these were finally selected as 'probable candidate peptides'. Of these nine 'probable candidate' peptides, using solid phase chemistry, two peptides; that is, InlC.1M and InlC.2M were synthesized on alanine MAP (multi antigenic peptide) core, while rest of the other linear peptides; that is, InlC.3L, InlC.4L, InlC.5L, InlC.6L, InlC.7L, InlC.8L and InlC.9L were synthesized by Wang resin method (Table 1).

Standardization of indirect enzyme-linked immunosorbent assay (ELISA) for InlC peptides

InlC peptide based Indirect ELISA was standardized employing checkerboard titration method. In brief, the plates were coated overnight at 4°C with different peptides (100 µl/well) having different concentrations (10000, 5000, 2500, 1250 and 625 ng/well). Later, the plates were incubated at 37°C for one hour and washed thrice with phosphate buffer sulphate with Tween (PBST). Unsaturated sites were blocked with 200 µl of 0.5% BSA fraction V in PBS and incubated at 37°C for 2 h in humidified chamber. Later the plates were washed thrice by PBST.

Test sera were diluted serially from 1:25 to 1:32000 in blocking buffer which was then added to the plates at 100 µl/well. Both positive and negative sera were included in the assay. The plates were incubated at 37°C for 2 h and then washed thrice with PBST. Anti- goat IgG HRP conjugate (Sigma) at a dilution of 1:10,000 was then added (100 µl/well) and further incubated at 37°C for 1 h. After incubation, the plate was washed thrice by PBST. The reaction was visualized by adding substrate solution at 100 µl/well having 6 mg OPD (sigma) in 10 ml of citrate buffer and 5 µl of 30% hydrogen peroxide. The plates were then incubated at room temperature for 15 min in dark. Finally the reaction was stopped by adding 50 µl of 2N H_2SO_4 stop solution and read at 492 nm using ELISA reader (EC, India).

A serum sample at the dilution of 1:100 with the positive-to negative (P/N) ratio of ≥ 2 was considered positive for anti-InlC peptide antibodies in standardized ELISA and p/n ≥ 1.8 to < 2 was considered as doubtful.

Serum samples

A total of 121 serum samples collected from apparently healthy slaughtered goats as well as sera from experimental goats were screened for antibodies against LLO and InlC peptides using standardized indirect ELISA described above.

RESULTS

In the present investigation, goats experimentally inoculated with live as well as heat - killed pathogenic strain of *L. monocytogenes* by oral and subcutaneous routes, respectively, were screened initially for rise in body temperature followed by clinical signs if any. Later the sera of these animals were studied to observe kinetics of antibody production against listeriolysin-O (LLO) and InlC peptides using standardized indirect ELISAs.

Out of five goats inoculated with pathogenic strain of *L. monocyotgenes*, three showed a sharp rise in body temperature (upto 104 to 105°F) within one to two days of infection which lasted for about three to five days and then gradually declined to normal by day eight post-infection (PI). However, one goat showed slight rise (102 to 102.5°F) in body temperature, whereas the fifth goat did not reveal any sign of rise in temperature. None of the infected goats revealed typical circling movement suggestive of listeriosis.

Isolation of the pathogen from five inoculated goats

Table 2. Shedding pattern (Faecal/nasal) of LM in of experimentally infected goats.

Goat number	Type of culture/route	Post-inoculation (day)						
		0	3	7	10	14	21	28
(G1/LM/V)	LM Live Oral	-	+	+	-	-	-	-
(G2/LM/V)	LM Live Oral	-	+	+	-	-	-	-
(G3/LM/V)	LM Live Oral	-	+	+	-	-	-	-
(G4/LM/V)	LM Live Oral	-	ND	ND	ND	ND	ND	ND
(G5/LM/V)	LM Live Oral	-	-	-	-	-	-	-
(G1/LM/K)	LM killed S/C	-	-	-	-	-	-	-
C1	Nil oral	-	-	-	-	-	-	-
C2	Nil s/c	-	-	-	-	-	-	-

ND - Not done

was attempted from their blood, rectal and nasal swabs taken at different intervals during the course (60 days) of the study (Table 2). The listeriae could not be isolated from blood in any of the infected goats, however, nasal and fecal swabs, revealed cultural positivity in case of three inoculated animals on day three and seven PI (Table 2). One of the infected goats suffered from diarrhea and died on day 60 PI. Pathological examination of the goat revealed enteritis and encephalitis; and the brain tissue smear showed Gram positive rods which were further confirmed by isolation and biochemical characterization. However, owing to liquefactive changes in brain, the presence of micro-abscesses could not be appreciated. The goat inoculated subcutaneously with killed culture of pathogenic *L. moncytogenes*, did not show any rise in the body temperature nor revealed the presence of the pathogen in the faecal and nasal sheddings during the study period (Table 2). Both goats kept as uninfected controls also had no signs of rise in the body temperature and did not reveal the presence of the pathogen in their sheddings (Table 2).

Kinetics of antibody production against listeriolysin-O (ALLO)

Prior to inclusion of goats in the study, all the goats intended for use in experimental study were tested for absence of antibodies against LLO (ALLO) using standardized indirect plate ELISA. Post infection, an appreciable sero-conversion against LLO was observed in four of five goats inoculated with *L. monocytogenes* with a positive to negative (p/n) ratio of ≥ 2 from day 14 onwards, which increased thereafter. The titer of the (ALLO) peaked during 21 to 28 days PI and then decreased gradually. ALLO titers remained detectable till the end of the experiment (upto 60 days) (Figure 1). However, ALLO could not be detected in the goat inoculated with heat killed culture of *L. monocytogenes* and also in uninfected control goats (Figure 1).

Kinetics of antibody production against Internalin-C (AInlC)

Before analyzing the kinetics of antibody production against InlC peptides, the nine 'probable candidate' synthetic peptides (InlC.1M to InlC.9L) were screened initially with sera of experimentally infected goats by standardized indirect ELISA. Of the nine InlC peptides, three peptides namely, InlC.1M, InlC.3L and InlC.7L showed significant sero-positivity, and therefore, finally named as 'candidate peptides'. Further, combinations of candidate peptides were made and referred to as "Mixotopes"- Mx1(InlC.1M and InlC.3L), Mx2 (InlC.1M and InlC.7L), Mx3 (InlC.3L and InlC.7L) and Mx4 (InlC.1M, InlC.3L and InlC.7L). These mixotopes combinations were screened by indirect ELISA using the sera collected from experimental animals. On analysis of results, it revealed that the Mx4 was found to be the best mixotope, amongst all tested individual peptides and their combination and its BLAST analysis showed specificity to InlC of *L. monocytogenes* and *L. ivanovii*.

On day zero; that is, the day on which goats were experimentally inoculated, the sera of all experimental goats (eight) were found to be negative for antibodies against InlC (AInlC) when tested by peptide (Mx4)-based indirect ELISA. Later, an appreciable sero-conversion was observed against InlC with a p/n ratio ≥ 2.0 in three out of five *L. monocytogenes* inoculated goats on day 14 PI, which increased thereafter, and attained peak by days 21 to 28 PI by peptide-based ELISA. Subsequently, the titres decreased gradually but remained detectable at non-significant levels upto day 40 PI (Figure 2). AInlC titer was not elicited in the goat inoculated subcutaneously with heat killed culture as well as in the goats kept as control (Figure 2).

Comparison between the ALLO-and AInlC- positivity

Antibodies against LLO (ALLO) and InlC (AInlC) in sera of goats inoculated with live *L. monocytogenes* were detected by indirect ELISA on day 10 to 14 PI, with peak titres on day 21 to 28 PI. Subsequently, the ALLO and AInlC titres showed a gradual decline and were non-significant on day 60 PI and day 40 PI, respectively (Figures 1 and 2). Significant titres of ALLO were observed in four out of five animals inoculated with live *L. monocytogenes* whereas, only three of these animals showed significant antibody titres against InlC. The goat inoculated with heat

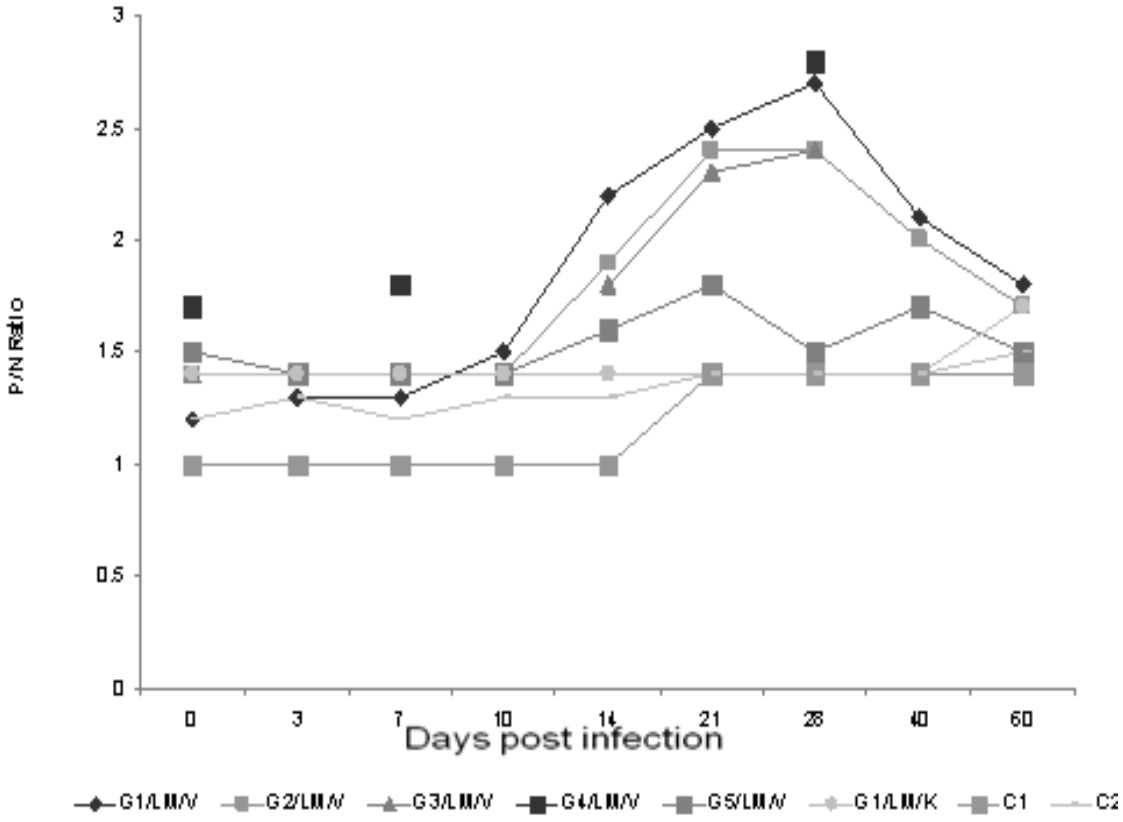

Figure 1. Kinetics of LLO antibodies in experimental goats by indirect ELISA. G: goat, LM: Listeria monocytogenes, V: viable/live, K: killed, C: control goats.

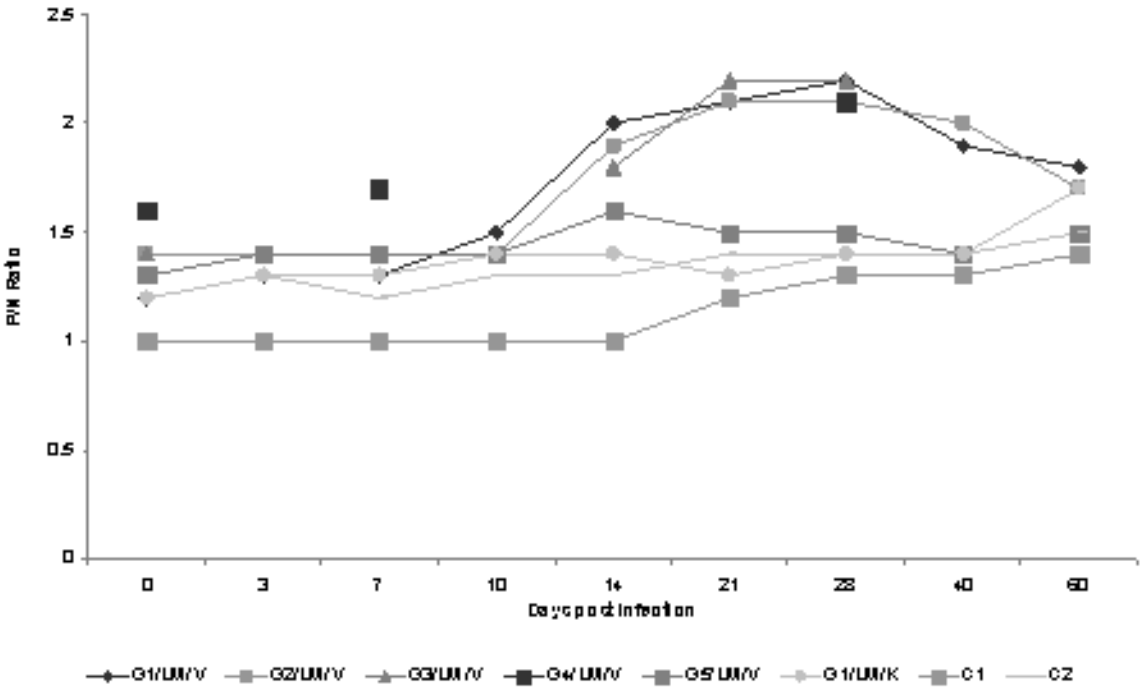

Figure 2. Kinetics of InlC peptide antibodies in experimental goats by indirect ELISA. G: goat, LM: Listeria monocytogenes, V: viable/live, K: killed, C: control goats.

Table 3. Efficacy of different ELISA's in apparently healthy goats (n=121).

Status	Criterion	No. positive	Percent positive
LLO+ve	p/n>2	23	19
LLO +ve (SLO adsorbed)	p/n>2	13	10.7
InlC Mixotope +ve	p/n>1.8	25	25.6
InlC Mixotope +ve	p/n>2	6	4.9
LLO +ve (SLO adsorbed) plus InlC Mixotope +ve	p/n>2	4	3.3

killed organisms as well as uninfected goats kept as negative controls did not reveal ALLO or AInlC titers in their sera (Figures 1 and 2).

Screening of field sera

On screening of sera from apparently healthy slaughtered goats (121) by the InlC-based ELISA (p/n ratio ≥ 1.8), InlC ELISA (p/n≥2), LLO-based ELISA (p/n≥2) and LLO-based ELISA (after adsorbption of sera with streptolysin) (p/n ≥ 2) revealed the seropositivity in 25.6, 4.9, 19 and 10.7% goats, respectively (Table 3). On comparison, an overall seropositivity of 3.3% was observed in indirect ELISA against InlC-(p/n ≥ 2) and LLO (After adsorption of sera with SLO). Although, InlC-based ELISA could detect seropositivity in two more cases, however, seven serum samples which showed positivity in LLO based ELISA (after SLO adsorption) were found negative in InlC-based ELISA.

DISCUSSION

To elucidate the role of antibodies in protection against listerial infection, studies aiming to identify and characterize new antibody targets are necessary. Under this context a study based on sequence analysis and western blot analysis identified eight *L. monocytogenes* proteins, three internalin members (InlA, InlD and InlC2) and five novel proteins of unknown function (designated IspA, IspB, IspC, IspD and IspE, respectively) as targets of humoral immune responses during listerial infection. These proteins were found to induce humoral immune responses during infection and thus opinioned to be useful candidates for serodiagnosis, vaccine and drug development (Yu et al., 2007). The candidate antigen, the InlC when employed as diagnostic antigen in recombinant InlC (rInlC) based ELISA gave promising results in detecting serological responses in experimental infected ewes (Zundel et al., 2007) whereas purified InlC earlier used in immunoblot have shown variable results in clinical cases of human Listeriosis (Grenningloh et al.,1997). Therefore in the present study we decided to use this protein in development of indirect based ELISA for serodiagnosis of listeric infections utilizing synthetic peptide based approach.

Oral dosing of pathogenic strain of *L. monocytogenes* caused no illness in goats except for mild pyrexia and diarrhea. In the present study, the recovery of *L. monocytogenes* from the brain of one goat suggested characteristic listeric encephalitis, which is in agreement with reported association of this pathogen with cases of encephalitis in goats (Yousif et al*.,* 1984; Oevermann et al*.,* 2010). In our study two goats failed to reveal an appreciable increase in body temperature and the presence of the pathogen in its faecal and nasal swabs. These intra-species variations might be due to the differences in the humoral and cellular responses of the experimentally infected animals against *L. monocytogenes* (Miettinen et al*.*, 1990; Bhanurekha et al*.,* 2006). Besides, killed culture of *Listeria* failed to elicit any clinical response (Yu et al*.,* 2007) which was concurrent with the results obtained in the present study. LLO, being a secretary protein which secreted only by live bacterial cells, therefore none of the goats inoculated subcutaneously with killed culture of pathogenic *L. monocytogenes* showed rise in temperature, pathogen isolation and ALLO, which is in accordance with similar observations reported in case of rabbits infected with *L. monocytogenes* (Yu et al*.*, 2007).

An appreciable ALLO was detected on day 14 PI, with peak between 21 to 28 days in case of four out of five *L. monocytogenes* inoculated goats and titres were detectable till 60 days PI in our study was in concordance with similar studies of (Bhanu Rekha et al., 2006; Miettinen et al., 1990).

In the present study, a significant sero-conversion with high levels of antibodies against InlC (AInlC) was observed in three out of five *L. monocytogenes* infected goats on day 14 PI which peaked on days 21 to 28 PI. These observations suggest an active listeric infection in these goats which showed initial appreciable rise in their body temperature and also shedding of the pathogen in their faecal/nasal samples till day 7 PI. Failure of the recovery of pathogen and absence of AInlC noticed in one *L. monocytogenes* - inoculated goat might be attributed to the intra-species variations in the immunological response or even complete absence of any serological response in animals given low infective doses, like that witnessed as sero-negativity of all the ewes in rLLO- and InlA-based ELISAs following their experimental infection with low doses (10^4 to 10^6 live organisms) of *L. monocytogenes* (Zundel et al*.*, 2007). Another possibility could be due to lower specificity or sensitivity of InlC peptide-based ELISA employed in our study, which might have failed to detect AInlC in this goat.

Unlike our study, wherein detectable titre of ALLO persisted for longer period (upto day 60 PI) in comparison to that of AInlC (upto day 40 PI) in goats experimentally infected with live *L. monocytogenes*, both type of antibodies (ALLO and AIrpA) remained detectable up to the end of study (day 48 PI) in ewes experimentally infected with *L. monocytogenes* (Zundel et al*.,* 2007). In terms of their efficacy, both the ELISAs (LLO-and InlC peptide-based) employed in our study were found to be specific for detecting the true negative animals; that is, animals inoculated with killed *L. monocytogenes* as well as uninfected controls.

It has been reported that the internalin A-related protein IrpA, also called internalin C is associated with the virulence of *L. monocytogenes*, and is a major protein target of the human humoral response to *L. monocytogenes* (Grenningloh et al., 1997). However, on comparison of SLO-adsorbed LLO-based ELISA (p/n ≥ 2) and InlC-based ELISA (p/n ≥ 2), respectively, an overall seropositivity (3.3%) was quite low. Besides, the latter test detected two more sera as positive for AInlC that were found negative for ALLO (SLO adsorbed sera). In contrast to these findings seven sera that were found positive for ALLO (SLO adsorbed sera), turned out to be negative for AInlC. These findings suggest that InlC-based peptide ELISA requires further improvement in order to make it more specific and sensitive through the use of mixotope(s) of InlC peptide(s) with other peptide(s) directed against more reliable virulence markers of listeric infection.

Results of experimental and spontaneous studies on listeric infection in goats reveal that antibodies against InlC protein and against LLO were elicited in goats following their experimental infection, and these were also detected in sera of apparently healthy goats by InlC peptide-based and LLO-based ELISAs, respectively. However, in the absence of an appreciable correlation between AInlC and ALLO, further improvement in ELISA is needed, particularly in terms of identification, synthesis and evaluation of appropriate synthetic peptide(s) for essential virulence markers of listeriae. These observations although are in contrast with that of an earlier report in ewes experimentally infected with *LM*, wherein rIrpA-based ELISA was claimed to show more promising results than LLO-based ELISA (Zundel et al., 2007), but at the same time, both the approaches were not identical for ideal comparison.

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Full Length Research Paper

Bacteriological evaluation and antimicrobial sensitivity test of common herbal concoctions in Ogbomoso metropolis

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Herbal concoctions are extracts from plants such as its bark, leaves, stems, roots, seeds and flowers. Plant ingredients were obtained from Oja-igbo market in Ogbomoso, Oyo State and were prepared in the form of concoctions (soup or drink made usually from ingredients after boiling) or infusions (soaking the plant material and allowing it to stand for varying lengths of time) and assessed for their microbial load and type of microorganisms present and also for the degree of resistance and sensitivity to selected antibiotics .The following bacteria were isolated from the herbal concoctions: *Micrococcus sendentarius, Corynebacterium renale, Clostridium tertium, and Clostridium butyricum.* Antimicrobial susceptibility screening of the isolated bacteria indicated multiple resistance to most commonly used antibiotics such as Ofloxacin (75%), Erythromycin (100%), Ciprofloxacin (100%), Clindamycin (100%), Gentamycin (75%), Cephalexin (100%), Cotrimoxazole (100%), Cloxacillin (100%), Ceftriaxone (100%) and Augmentin (100%). Only Ofloxacin and Gentamycin had (25%) sensitivity rate. The microbial quality of herbal concoctions should be of great importance to the community in order to reduce harm to the consumers and spread of resistance strains.

Key words: Antimicrobial, herb concoctions, susceptibility, resistance, Ogbomoso.

INTRODUCTION

Plants produce a diverse range of bioactive molecules, making them rich source of different types of medicines. Most of the drugs today are obtained from natural sources or semi synthetic derivatives of natural products used in the traditional systems of medicine (Sukanya et al., 2009). Medicinal plants are finding their way into pharmaceuticals, cosmetics, and neutraceuticals. In pharmaceutical field medicinal plants are mostly used for the wide range of substances present in plants which have been used to treat chronic as well as infectious diseases (Okigbo et al., 2009).

Plants have been utilized as a source of medicine for thousands of years and continue to play an important role globally in primary health care, mostly in developing countries (Balunas and Kinghorn, 2005). The use of medicinal plants is increasing because people believe they are safe for human consumption.

Traditional medicines are used not only for primary health care of the poor in developing countries, but also in countries where conventional medicine is predominant in the national health care system (Lanfranco, 1999). Herbal medicines serve the health needs of about 80%

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of the world's population, especially for millions of people in the vast rural areas of developing countries; more than 65% of the global population uses medicinal plants for their primary health care needs (WHO, 2001).

Antibiotic resistance has become a global concern in recent years. This problem is of great significance especially in developing countries because infectious diseases are one of the major causes of mortality in these countries. Due to a rapid increase in the rate of infections, antibiotic resistance in microorganisms and due to side effects of synthetic antibiotics, medicinal plants are gaining popularity over these drugs (Babu and Subashree, 2009).

Medicinal plants have been found useful in the cure of a number of diseases including bacterial diseases. Medicinal plants are a rich source of antimicrobial agents (Mahesh and Satish, 2008). Although medicinal plants produce slow recovery, the therapeutic use of medicinal plant is becoming popular because of their lesser side effects and low resistance in microorganisms (Seyyednejad and Motamedi, 2010).

A herb is a plant or any part of a plant valued for its' medicinal, aromatic, or savory qualities (Bodeker et al., 2005; Bisset, 1994). Herbs can be viewed as any biosynthetic laboratory ingredients, producing a number of chemical compounds. Herbal remedies or medicines consist of portions of plants or unpurified plant extracts containing several constituents, which often work together synergistically. According to the World Health Organization (WHO), ''Herbal Preparations'' contain plant parts or plant material in the crude or processed state as active ingredients and may contain excipients (foreign substances) (WHO, 1998; Busse, 1999). Herbal preparations are comminuted or powdered plant material, extracts, tinctures, fatty or essential oils, expressed juices, processed resins or gums and so forth prepared from different plant parts such as roots, bark, stems, leaves, and fruits whose production involves a fractional, purification, or concentration process (Evans, 1989; Evans, 1996).

There is also an increase in infectious diseases worldwide caused by both drug resistance; and lack of sufficient affordable medicine for people living in poor communities. Infectious diseases represent an important cause of morbidity and mortality among the general population, particularly in developing countries.

Microbial infections have posed a health problem throughout the world, and plants are a possible source of antimicrobial agents (Burapadaja and Bunchoo, 1995; Adenisa et al., 2000). Medicinal plants contain active principles which can be used as an alternative cheap and effective herbal drug against common bacterial infections. During the past decade, medicinal plants have been extensively used for medical treatments. They have also been traditionally utilized in the history including some of their biological activities which have been scientifically characterized. Product quality is obviously one of the major criteria that could affect not only the efficacy of the herb but also the safety of patients or consumers.

Ogbomoso, a city in Oyo state, South-western part of Nigeria are endowed with wide varieties of indigenous medicinal plants. These plants are used by the local herbalists for treatment of a number of diseases, both bacterial and non-bacterial type. Medicinal plant materials normally carry a large number of microbes originating from the soil and handlers. Microorganisms of various kinds are normally adhered to leaves, stem, flowers, seeds, and roots. Additional contaminants may also be introduced during harvesting, handling, and production of various herbal remedies since no conscious efforts are made to decontaminate the herbs other than washing them (Sofowora et al., 1982). Therefore, bacteriological contents in herbal concoctions should be evaluated. Microbial contaminations are frequently involved in herbal products since all products come from plants. In this study, the bacteriological and antimicrobial sensitivity assessment of bacterial of herbal concoctions prepared locally for the treatment of malaria, fistula, convulsions, and skin rashes was investigated.

MATERIALS AND METHODS

Sample collection

All the different plant ingredients for the preparation of the herbal concoction for various treatments were obtained from different sellers in Oja-igbo market in Ogbomoso, Oyo state, Nigeria, as shown in Table 1. Based on the treatment, the samples were collected differently in separate sterilized containers. Some of the herbal ingredients were prepared in the form of concoctions (soup or drink made usually from ingredients after boiling) or infusions (soaking the plant material and allowing it to stand for varying lengths of time).

Microbiological analysis

Materials used

The materials used for this experiment included: the various herbal mixtures, autoclave, Petri-dishes, cotton wool, nutrient agar, Potato dextrose agar, inoculating loop, distill water, slant bottles, beakers, measuring cylinders, jars, electrical cooker, measuring scale, aluminum foil, spatula, spirit lamps, test tubes.

Sterilization

All the test tubes, beakers and glass wares were placed in the autoclave and then subjected to a temperature of 121°C for 15 min. After sterilization, they were allowed to cool and kept for use.

Preparation of culture media

Nutrient agar

This is a microbiological growth medium commonly used for the routine cultivation of non-fastidious bacteria. The medium was prepared based on manufacturer's directives. Depending on the

Table 1. The botanical and common names, parts used and family of the plants used in the preparation of the herbal concoctions and the family which they belong.

Usage of herbal concoction/ botanical names	Common/local names (Y-Yoruba; H- Hausa; I-Igbo)	Parts used	Family
Malaria (Iba)			
Enantiachlorantha	Awopa (Y), yellow wood	Bark	Annonacease
Citrus aurantifolia	Osanwewe (Y), lime	Juice	Rutaceae
Cymbopoqoncitratus	Ewe tea (Y), Lemon grass	Leaf	Poacease
Maqniferaindica	Ewe mangoro (Y), Mango	Leaf	Anacardiaceae
Azadirachta indica	Dogonyaro (H), Neem tree, Aforo-oyingbo (Y),	Leaf	Meliaceae
Pile (Jedijedi)			
Sabiceacalycina	Ogan (Y)	Bark	Rubiaceae
Lanneawelwitschii	Orira (Y)	Bark	Anacardiaceae
Aristolochiaalbida	Akoigun (Y)	Leaf	Aristolochiaceae
Lophiralanceolata	Panhan pupa/funfun (Y)	Bark	Ochnaceae
Syzygiumaromaticum	Konofuru (Y), clove	Fruit	Myrtaceae
Tetrapleuratetraptera	Aidan (Y)	Fruit	Mimosaceae
Convulsion (Giri)			
Ocimum gratissimum	Efirin (Y), Nchianwu (I)	Leaf	Lamiaceae
Black alum	Omiroro	ND	ND
Skin rashes (Narun)			
Lophiraalata	Uda, pahan (Y),	Roots	ND
Ceibapentandra	Poripola(Y)	Stem	ND
Pergulariadaemia	Eseatufa, Kole-agbe (Y)	ND	ND

number of plates to be prepared, certain grams of the powder was dissolved in specific litre of distilled water in a beaker or jar, mixed vigorously and heated on an electrical cooker (hot plate) till the powder was completely dissolved. Thus was followed by sterilization at 121°C for 15 minin the autoclave.

Culturing of organisms

Serial dilution

The concentration of the original solution and the desired concentration will determine how much the dilution needs to be and how many dilutions are required and the total volume of solution needed. Using a sterile pipette, 1 ml of each of the prepared herbal concoctions were transferred to separate sterile test tube containing 9 ml of sterile distilled water and shaken to obtain 10^{-1} dilution of sample. 1 ml of 10^{-1} dilution of sample was taken into sterile test tube containing 9 ml sterile distilled water to obtain further dilution of 10^{-2}. The procedure was continued until the desired diluents were derived.

Pour plate method

Media pouring is defined as the process by which sterile media are being poured into sterile Petri dishes and this was done in the inoculating room in the laboratory. Before pouring of the media, the air conditional was put on and the working bench/ slabs were sterilized to avoid contamination.

Isolation and preservation of isolates

After inoculation and incubation of isolates to ensure growth of associated bacteria, colonies which develop on the plates were counted and recorded as colony forming unit per gram of the (Cfu g^{-1}) sample. From the mixed cultures, distinct colonies were picked and streaked unto a freshly prepared media under aseptic conditions to obtain pure cultures. All the pure cultures for bacterial isolates were kept on nutrient agar slants and stored in the refrigerator as working and stock cultures.

Characterization of isolates

Identification and taxonomic studies were carried out on the purified isolates on the basis of their cultural, morphological, biochemical, and physiological characteristics.

RESULTS

The results of the bacterial isolates present in the four samples of the herbal concoctions indicated that all the bacterial isolates obtained were Gram positive and they all appeared in rod form except for the bacteria-*Micrococcus sedentarius* which was isolated from Agboiba that is coccoid in shape. The biochemical analysis which included test for catalase, oxidase, indole, motility, glucose, xylose, sucrose and so on indicated diversity among the different isolated bacteria. Table 2 shows the specific bacterium isolated from the various samples of herbal concoction. The bacteria *Clostridium* was isolated from two herbal concoctions but the species varied; *Clostridium tertium* was isolated from the concoct-tion used in the treatment of fistula (Agbojedi) while *C. butyricum* was isolated from the concoction used in the treatment of convulsion (Agbogiri). Other bacteria isolated

Table 2. Biochemical Characteristics of the Bacterial Isolates from the Four Herbal Concoctions.

Sample	Catalase test	Oxidase Test	Indole test	Motility test	Methyl red test	Vogesproskauer	Urease activity	Citrate utilization	Starch hydrolysis	Gelatin hydrolysis	Casein hydrolysis	NO_3 reduction	Spore test	Coagulase test	Glucose	Xylose	Lactose	Sucrose	Maltose	Mannitol	Raffinose	Arabinose	Sorbitol	Sorbose	Salicin	Fructose	Probable organisms
Agbo Iba	+	–	–	–	–	+	–	–	–	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	***Micrococcus sendentarius***
Agbo Narun	+	–	–	–	–	+	+	–	–	+	–	–	–	–	+	–		–	+	–	–	–	–	–	–	+	***Corynebacteriumrenale***
Agbo Jedi	+	+	–	+	–	–	–	–	–	+	–	+	+	–	+	–	+	+	–	–	–	–	–	–	+	–	***Clostridium tertium***
Agbo Giri	+	+	–	+	–	–	–	–	–	–	–	+	+	–	+		+	+	–	–	–	–	–	–	+	–	***Clostridium butyricum***

+ = positive; - = negative.

Table 3. Antimicrobial Test for the Bacterial Isolates in Millilitre (mm).

Samples	Organism	OF	E	CIP	CD	GN	CX	CO	AP	FX	AU
Agboiba	*Micrococcus sedentarius*	R	R	R	R	R	R	R	R	R	R
Agbonarun	*Corynebacterium renale*	R	R	R	R	R	R	R	R	R	R
Agbojedi	*Clostridium tertium*	R	R	R	R	R	R	R	R	R	R
Agbogiri	*Clostridium butyricum*	6.0	R	R	R	7.0	R	R	R	R	R

OF, Ofloxacin; E, Erythromycin; CIP, Ciprofloxacin; CD, Clindamycin; GN, Gentamycin; CX, Cephalexin; CO, Cotrimoxazole; AP, Cloxacillin; FX, Ceftriaxone; AU, Augmentin; R, Resistance.

from concoctions used in the treatment of malaria and skin rashes are *Micrococcus sedentarius* and *Corynebacterium renale* respectively.

Table 3 shows the result of the antimicrobial test of the isolated bacteria. Virtually all the micro-organisms were resistant to the antibiotics except for *C. butyricum* which was susceptible to Ofloxacin (OF, 5 µg) and Gentamycin(GN, 10µg) within the range of 6.0-7.0 mm. Other antibiotics which the bacteria were resistant to are; Erythro-mycin (E, 10 µg), Ciprofloxacin (CIP, 5 µg), Clin-damycin (CD, 10 µg), Cephalexin (CX, 30 µg), Cotrimoxazole (CO, 50 µg), Cloxacillin, (AP, 30 µg), Ceftriaxone (FX, 30 µg) and Augmentin (AU, 30 µg).

DISCUSSION

Results obtained from this research shows that the common herbal concoction contained different bacterial isolates. The samples were all water based. Majority of the herbal ingredients are always exposed not following good manufacturing practices and this accounts for the high rate of bacterial contaminants that renders the concoct-tions virtually fearful especially when consumed excessively with improper prescription.

C. tertium is a spore forming anaerobic Bacillus found in the gut of many animal species including humans (Miller et al., 2001). This bacterium is a non-toxin producing, aerotolerant, non-histotoxic and non-lipolytic specie. *C. tertium* has traditionally

been considered non-pathogenic (Ray et al., 2003), but it is found associated with meningitis, septic arthritis and pneumonia (Ferrell and Tell, 2001). *Clostridium butyricum* is a strictly anaerobic endospore forming, butyric acid producing Bacillus (Seki et al., 2003). *C. butyricum* is a soil inhabitant and uncommonly reported as a human pathogen (Meng et al., 1997). *Corynebacterium renale* has not been reported as human pathogen but a pathogenic veterinary bacterium that causes cystitis and pyelonephritis in cattle. The bacterium is sensitive to the majority of antibiotics, such as Penicillin, Cephalosporin, Tetracycline and Chloramphenicol. *M. sedentarius* is also a soil inhabitant. This organism cannot by itself initiate infection but is an opportunistic pathogen particularly in hosts with immune-compromised immune system (Smith et al., 1999).

Conclusion

This present study has shown that there are varieties of microorganism present in our various herbal concoctions which could have resulted from contaminated soils, plants and its products, preparation processes, quality of water, containers and processing equipment. However, these microorganisms exhibit multi-resistance to many antibiotics. Since herbal concoctions are mainly prepared for human consumption, there is a very high chance of passing the antibiotics resistant microorganisms into the human ecosystem. This poses a great danger to human health. Since herbal concoctions are prepared using varieties of medicinal plants which contain active constituents that are cheap and effective against common bacterial infections. Therefore, it is suggested that proper hygienic conditions should be maintained in all preparation processes starting from plant collection, processing, packaging and storage. There is need for mass education to enlighten the public on excessive consumption of herbal products and other drugs since many microorganisms isolated from this study are resistant to most of the common antibiotics. Also, herbal practitioners should be encouraged to send their products regularly to laboratories for quality assessment to ensure consistency and quality before marketing.

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Full Length Research Paper

Effect of ultraviolet-B radiation on arbuscular mycorrhizal colonization of two rangeland weeds

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Our experiment aimed at assessing the influence of ultraviolet-B (UV-B) radiation on the growth and colonization of roots of two invasive rangeland species: houndstongue (*Cynoglossum officinale* L.) and downy brome (*Bromus tectorum* L.) with arbuscular mycorrhizal fungi (AMF). As a result, after 13 weeks of growth in the presence of 8 kJ*m^{-2} UV-B radiation (estimated as 23% of ozone layer depletion), a significant reduction of houndstongue's lamina length, leaf area, shoot and root dry mass was noted. As for downy brome, only the stem length and shoot dry mass were negatively affected. Nevertheless, the intensity of UV-B radiation downy brome developed a similar number of inflorescence. For both species in the presence of elevated UV-B$_{BE}$ radiation, a significant reduction in the AMF colonization was noted, especially for hyphae and arbuscules. In conclusion, the elevated UV-B radiation may affect the performance of both weeds and relate with their competition abilities.

Key words: Arbuscule, downy brome, growth, houndstongue, hyphae.

INTRODUCTION

Continuous reduction of the earth's protective ozone layer thickness and as a result, an increase in the amount of solar ultraviolet-B radiation (UV-B: 280 - 320 nm) in the atmosphere is observed. The increased levels of UV-B radiation pose adverse effects on organisms, including plants (Ballaré et al., 2010). As a result of such action, the reduction in biomass accumulation, growth disorders and leaves' curling could be observed (Furness et al., 1999), as well as changes in the physiological parameters (Jansen et al., 1998), such as the increase in UV-B absorbing compounds (Ren et al., 2010). Different plant species are exposed to diverse levels of sensitivity of the increased UV-B radiation. In the rangeland phytocenosis composed of both grasses and weed species, this differentiated sensitivity may be of importance, as can either promote (Smith et al., 2000) or inhibit the growth and development of cultivated and weed species (Dai and Upadhyaya, 2002; Furness et al., 1999; Stokłosa et al., 2012). As a result, not only aboveground but also the below ground competition between species is affected (Zaller et al., 2002).

UV-B radiation inhibits also the performance of beneficial microbes such as bioherbicidal fungi, *Clonostachys rosea* (Costa et al., 2012) or the conidial growth of freshly harvested fungal isolates (Le Grand and Cliquet, 2013) or plant symbiotic microbes. Up till now, little is known about how exposure to UV-B radiation affects rhizosphere microbes. Rhizosphere organisms, which are fed primarily by root-derived substrates, fulfill diverse functions such as mineralization, immobilization, decomposition, pathogeneity and improvement of plant nutrition (Klironomos and Allen, 1995). Among them is a special

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Table 1. Photosynthetically active radiation (PAR) course (mmol m^{-2} s^{-1}) for different UV-B treatments during the experiment (measured weekly around noon).

Month		December		January		February		March		April		Average
Overcast		sunny	cloudy	sunny	cloudy	sunny	cloudy	sunny	cloudy	sunny	cloudy	
UV-B levels	0	156	50	158	68	165	134	186	155	441	150	166
	4	121	49	136	63	146	124	224	153	420	128	156
	6	136	52	150	70	172	134	258	147	433	146	170
	8	149	51	157	65	166	131	215	160	526	153	177
Average		140.5	50.5	150	66.5	162	131	221	154	455	144	
LSD		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

role played by arbuscular mycorrhizal fungi (AMF), which are responsible for improving the uptake of phosphorus from soils (Ahabior and Hirata, 2003). We hypothesized that stress, induced by increased UV-B radiation levels, may affect this symbiosis. Recent studies of Regvar et al. (2012) proved that UV-B radiation decreases the number of microsclerotia of fungi colonizing buckwheat (*Fagopyrum esculentum*) and tatar buckwheat (*Fagopyrum tataricum*).

In this study, we tested the response in growth and AMF colonization of roots of two invasive weeds occurring in the grassland area: dicotyledonous houndstongue (*Cynoglossum officinale* L.) and monocotyledonous downy brome (*Bromus tectorum* L.) (DiTomaso, 2000). Houndstongue is an allelopathic species, which causes external skin irritation of cattle due to the seeds that stick to the nutlet and also gastric problems (Upadhyaya and Cranston, 1991). Downy brome is an annual, invasive grass, of largely extended area of spread in the recent years, especially in Canada and USA (Vaillant et al., 2007). Both studied weeds are of economic importance, due to the adverse effects on livestock (houndstongue) and a depletion of valuable pasture flora (downy brome).

The research aimed at evaluating the effect of UV-B radiation on 1) growth of two rangeland weed species and 2) their root colonization with arbuscular mycorrhizal fungi.

MATERIAL AND METHODS

Seed source and plant culture

Two rangeland weed species: houndstongue (*C. officinale* L.) and downy brome (*B. tectorum* L.) were collected from field populations in southern British Columbia, Canada. Three pre-germinated seeds per pot were transferred into four pots (9.0 × 9.0 × 10.0 cm) filled up with non-sterilized top layer of soil (66.1% sand; 24.9% silt; 9.0% clay; 8.4% organic matter; 4.14% C; 0.24% N; 115.8 ppm P_2O_4; pH 5.90). Germinated plants were later thinned to one per pot. Pots were placed under the UV-B treatments in the greenhouse and plants were allowed to grow for 13 weeks. There were 2 sets of experiment, between December 2009 and April 2010.

Supplemental lighting (16-h photoperiod) was supplied by high-pressure sodium lamps. Photosynthetically active radiation (PAR) was measured weekly at noon with a LI-COR LI-185B portable light meter (Table 1).

Ultraviolet-B radiation treatments

UV-B treatments were provided by ten UVB-313 40W fluorescent tubes, installed 1.10 m above the greenhouse bench, in a 1.20 m long × 1.20 m wide × 1.25 m high frame enclosed on all four sides with Mylar film (Type D, 0.127 mm thick). Within this frame, smaller frames (60.0 × 32.0 × 40.0 cm high) were covered with either a layer of Mylar film, or one, two or three layers of cellulose acetate film (diacetate type, 0.127 mm). Mylar film absorbs all UV-radiation below 320 nm and cellulose acetate below 290 nm (Barnes, 1988). Different number of cellulose acetate layers provided different levels of UV-B radiation, whereas Mylar treatment was employed to simulate the ambient greenhouse condition.

UV-B radiation (290 - 320 nm) was measured in single nanometer increments using an International Light IL 1700 Radiometer interfaced with an IL782A double-slit monochromator. Single nanometer readings were taken in an experimental chamber covered with opaque black plastic to exclude interference from visible light. Biologically effective UV-B (UV-BBE) radiation was estimated from these readings using Caldwell's (1971) generalized plant damage action spectrum normalized to 300 nm. Daily UV-B exposure for 8 h, centered on solar noon, provided approximately 0 (control), 4, 6 and 8 kJ*m^{-2} of UV-BBE radiation in frames covered with a layer of Mylar film, and three, two, or one layer(s) of cellulose acetate film, respectively. The applied UV-B radiation refer to the zero, field-ambient (4 kJ*m^{-2}), 13% (6 kJ*m^{-2}) and 23% (8 kJ*m^{-2}) of ozone layer depletion.

Growth and morphological response to UV-B radiation

After 3 months of growth experiment was terminated plants taken for measurements. For dicotyledonous houndstongue leaf number was counted, also length of petiole and lamina was measured along with leaf area. For monocotyledonous downy brome the length of main stem and panicle was taken, tillers number and spikelets number per plant was counted. Roots and shoots were dried at 50°C for 72 h and weighed.

Mycorrhizal colonization measurements

The intracellular structures of AMF naturally occurring in the tested soil were tested in this experiment. Magnified intersections method (McGonigle et al., 1990), was used to determine the proportion of root length in which arbuscles, vesicles or hyphae were present. For this purpose roots were cut into pieces of 1 cm long, cleared in 10% KOH (w/v), acidified with 5% HCl (v/v) and stained in 0.05% trypan blue. AMF colonization of roots was quantified using a compound Leica microscope (100×). 1000 intersections were examined per roots of single plant. The arbuscular colonization

Table 2. Morphological traits of houndstongue (*C. officinale* L.) growing under different levels of UV-BBE radiation, n = 8.

Trait	UV-B_{BE} level (kJ*m^{-2})				±SE	P level
	0	4	6	8		
Leaf number	5.3	4.1	4.5	4.3	0.38	0.1
Lamina length	9.9	9.8	8.5*	8.2*	0.31	<0.0001
Stalk length	4.9	5.0	5.3	4.7	0.22	0.4
Leaf area	28.2	27.1	25.5	19.5*	1.25	<0.0001
Shoot dry mass	1.0	0.9	0.6	0.5*	0.06	<0.0001
Root dry mass	2.0	1.4	1.1*	0.9*	0.13	<0.0001

*Denotes significant difference, as compared to control (0).

Table 3. Morphological traits of bromegrass (*B. tectorum* L.) growing under different levels of UV-BBE radiation, n = 8.

Trait	UV-B_{BE} level (kJ*m^{-2})				±SE	P level
	0	4	6	8		
Stem length	36.4	31.2	29.6	23.7*	1.06	<0.0001
Panicle length	13.2	13.6	14.3	13.4	0.41	0.4
Spikelets number	106.6	121.9	89.5	80.3	11.48	0.1
Shoot dry mass	1.4	1.1*	1.3	1.1*	0.03	<0.0001
Root dry mass	1.1	1.2	1.0	1.0	0.09	0.4

*Denotes significant difference, as compared to control (0).

(AC) and vesicular colonization (VC) were calculated by dividing the count for the 'arbuscules' and ' vesicles' categories respectively by the total number of intersections examined. Hyphal colonization (HC) was calculated as the proportion of non-negative intersections, according to McGonigle et al. (1990).

Both, the UV-B radiation on the plant growth as well as AMF analyses were carried out on the same plant material. There were 8 plants/biological replicates used in the statistical analysis (4 plants per one set, with two sets of a whole experiment). The analysis was carried out using STATISTICA 10 software (StatSoft 2011). One way ANOVA was applied to the tested objects. Significant differences between analyzed objects were assessed at $p \leq 0.05$.

RESULTS

Influence of UV-B radiation on growth of downy brome and houndstongue

Since there were no significant differences in the PAR radiation between different UV-B treatments, it can be assumed, that all the differences in the growth and development of both tested species are due to the reaction to the diversified UV-B levels (Table 1). After 13 weeks of growth, houndstongue was in a vegetative phase of growth, whereas downy brome was in the phase of flowering. Both tested species turned to be sensitive to the highest applied UV-B_{BE} dose (8 kJ), at the same time monocotyledonous downy brome was less susceptible, in comparison with dicotyledonous houndstongue. Two lower doses of UV-B_{BE} did not influence the growth and morphological features of both tested species.

Downy brome plants growing under elevated UV-B_{BE} conditions developed significantly shorter stems, by about 35%, as compared to plants growing under the UV-free conditions (Table 2). At the same time, the panicle length remained unchanged, regardless of UV-B stress. Shorter downy brome plants accumulated significantly less biomass in the aboveground parts (by about 23%, in comparison with the control), still the root biomass was not influenced by UV-B treatment. After 3 months of growth, downy brome plants were in the phase of flowering, so seeds were not developed yet. Still, it was possible to count the number of spikelets which, even though not significantly different, still were decreased by elevated UV-B levels by about 25% as compared to plants growing without UV-B light (Table 2).

In the presence of increased UV-B radiation (8 kJ), houndstongue plants developed insignificantly lower number of leaves (by about 20%), as compared to plants growing in UV-free conditions (Table 3). At the same time, houndstongue's leaves growing under 8 kJ of UV-B_{BE} had significantly shorter lamina (by about 18%) and significantly smaller leaf area (by about 31%), whereas stalk length was not affected. All UV-B induced changes in the morphology of houndstongue influenced shoot and root dry mass, which was significantly lower in the presence of 8 kJ of UV-B_{BE}, by about 45 and 55%, respectively (Table 3).

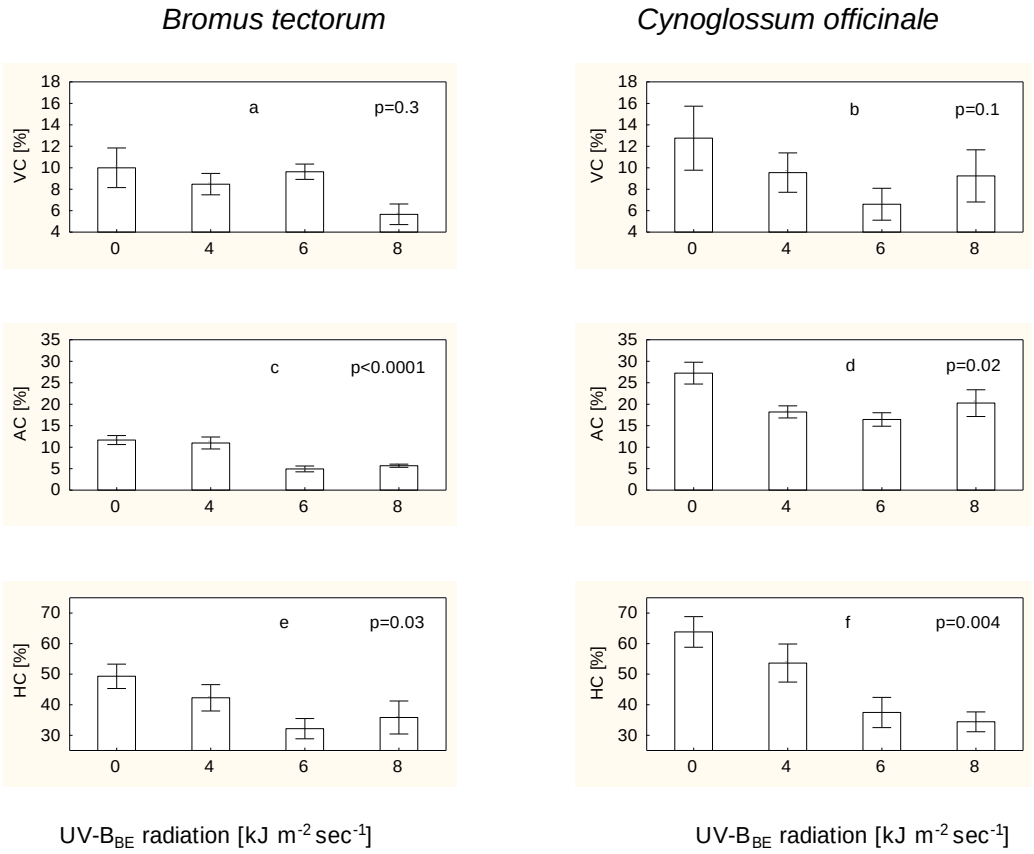

Figure 1. Arbuscular mycorrhizal colonization of downy brome and houndstongue roots growing in the presence of different levels of UV-BBE radiation. VC- vesicule colonization, AC- arbucule colonization, HC- hyphae colonization.

Influence of UV-B radiation on arbuscular mycorrhizal colonization

Houndstongue roots were more intensively colonized by mycorrhizae fungi, as compared to downy brome roots (Figure 1). The highest percentage of colonization with all intracellular fungi structures (hyphae, arbuscules and vesicles) was observed in the controlled, UV-free conditions. The percentage of roots colonized with hyphae in the control conditions was 63% for houndstongue and 50% for downy brome (Figure 1e and f). Applying UV-B stress caused significant decrease in the hyphae and arbuscules colonization of roots of both species. The higher doses of UV-B$_{BE}$ (6 and 8 kJ) caused reduction of hyphae by about 20 and 17% for downy brome (Figure 1e) and by about 42 and 47% for houndstongue (Figure 1f). In the case of arbuscules colonization, the reductions were by about 58 and 50% for downy brome (Figure 1c). The amount of arbuscules formating on houndstongue roots turned to be significantly susceptible to even the lowest UV-B$_{BE}$ treatment, and the reductions observed were: 33, 41 and 26% for 4, 6 and 8 kJ, respectively (Figure 1d). The vesicles colonization, remained significantly unchained regardless of UV-B$_{BE}$ treatment, although a visible decrease was noted for both hounstongue and downy brome grown under UV-B$_{BE}$ (Figure 1a and b).

DISCUSSION

Both species tested in the experiment showed a different susceptibility to UV-B radiation. As expected, more susceptible in terms of growth and development turned to be dicotyledonous houndstongue. Monocotyledonous weeds are generally less susceptible to UV-B radiation as compared to dicotyledonous species (He et al., 1993). The highest dose of UV-B, 8 kJ, affected all, except leaf number and stalk length, tested traits of houndstongue. The most susceptible turned to be leaf lamina length as well as leaves area. Our findings are similar to those of Furness et al. (1999), who studied properties of houndstongue seedlings in the presence of UV-B of 7 - 11 kJ*m^{-2} and observed a reduction of leaf, stem and root fresh weights, leaf area and leaf: shoot ratio and increase in shoot dry matter content, specific leaf weight and leaf greenness. Downy brome was less susceptible to UV-B

radiation and after 3 months of growth a reduction of stem length and shoot biomass was only noted. At the same time, the number of downy brome inflorescence per plant was not affected, suggesting that this species may still produce seeds under the UV-B radiation stress, which can add to its invasiveness properties and grasses plasticity, similarly to *Deschampsia antarctica* studied by Day et al. (1999). On the other hand, Dai and Upadhyaya (2002) have proved, that seedlings of downy brome express susceptibility to much more elevated UV-B radiation of 11 $kJ*m^{-2}$ at the germination phase (during 10 days of germination process), which can influence the later competitive abilities of this species.

Two structures of arbuscular fungi, associated with both houndstongue and downy brome, were significantly reduced by the elevated amounts of UV-B radiation: hyphae and arbuscules. Our findings are confirmed by Klironomos and Allen (1995), who observed a reduction of mycorrhizal symbiosis between *Acer saccharum* in the conditions of increased UV-B radiation and shift to a saprobe/pathogen system. Similar findings for the grassland communities in the five-year-long field observations were reported by van de Staaij et al. (2001). We also observed a strong reduction in the formation of vesicles in both species but, due to the large differences between single samples, this effect was insignificant. As a result of such changes, it is highly possible, that both species will have less ability to absorb minerals from the soil, especially phosphorus. This in turn can possibly lead to the reduced growth of both, shoots and roots, especially for houndstongue, and can have the influence on both species' competition abilities.

Summing up, the results of our study show, that under the elevated UV-B radiation (8 $kJ*m^{-2}$) both weeds demonstrate the differential growth responses. Also, the increased levels of UV-B radiation cause a significant reduction in the hyphae and arbuscule formation of mycorrhizal arbuscular fungi associated with both downy brome and houndstongue. Both findings may have significant implications for invasive species ability to compete with each other and with associated rangeland species. A further research should be undertaken to assess the actual competitive abilities of houndstongue and downy brome in the presence of grassland species in the conditions of the elevated UV-B radiation.

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Full Length Research Paper

Effect of *Pediococcus* sp., *Pediococcus pentosaceus*, inulin and fulvic acid, added to the diet, on growth of *Oreochromis niloticus*

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Lactic acid bacteria (LAB) were isolated from the intestine of *Oreochromis niloticus* and its effect, along with that of inulin and fulvic acid, on growth of the same species was determined. Characterization of the isolates was performed through assessing the Gram stain, morphology, cell arrangement, hemolytic activity, antagonism, growth, hydrophobicity, extracellular enzyme activity, and counting of colony forming units (CFU), to select those microorganisms with probiotic potential. The selected isolates were identified at the molecular level by the amplification of 23S and 5S ribosomal genes. Treatments were performed in triplicate in plastic tanks with 600 l of filtered fresh water and 12 fish per tank (1.3 ± 0.12 g): I) Control (commercial diet); II) LAB (5×10^4 CFU/g diet) + 2.5 g inulin + 2.5 g fulvic acid/kg diet; III) LAB (2.5×10^5 CFU/g diet) + 2.5 g inulin + 2.5 g fulvic acid/kg diet; IV) LAB (5×10^5 CFU/g diet) + 2.5 g inulin + 2.5 g fulvic acid/kg diet. We selected three LAB strains, belonging to *Pediococcus pentosaceus* (LAB 37 and LAB 1-6) and *Pediococcus* sp. (LAB 35), with probiotic potential. Growth in weight of individuals of treatment III was significantly higher than the control group. Lactic acid bacteria, inulin, and fulvic acid were appropriate growth-stimulating additives for cultured *O. niloticus* in the concentrations of treatment III.

Key words: Fulvic acid, *Oreochromis niloticus*, inulin, *Pediococcus pentosaceus*.

INTRODUCTION

Aquaculture requires quality diet with high protein content and complementary additives to maintain healthy organisms and to favor the growth of the animals. Some of the most used additives to promote growth include hormones, antibiotics, ionophores, and some salts (Klaenhammer, 1988; Fuller, 1994; Mohapatra et al., 2012). The inappropriate use of these growth promoters can have adverse effects on the animal and on the end consumer, and can promote resistance to pathogenic bacteria, as occurs with the use of antibiotics (Lara-Flores et al., 2003; Mohapatra et al., 2012).

Based on the aforementioned, there is a great interest in the use of natural feed additives such as probiotics to improve the resistance to diseases, water quality and/or growth of cultivated organisms (Verschuere et al., 2000; Nayak, 2010; Mohapatra et al., 2012). Probiotics have

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beneficial effects on the host due to the changes in the microbial community related to the host and the environment through an improvement in the utilization of the food or in its nutritional value, or by improving the host's response to disease or the quality of its environment (Verschuere et al., 2000; Balcázar, 2002; Balcázar et al., 2008; Zhou et al., 2010a; Mohapatra et al., 2012). Among the organisms commonly used as probiotics in fish are lactic acid bacteria (LAB) like *Lactobacillus plantarum* and *Lactobacillus fructivorans*, which have been tested in *Sparus aurata* (Carnevali et al., 2004), *Lactobacillus* sp. in ornate fish *Carassius auratus* and *Xiphophorus helleri* (Abraham, 2008), *Enterococcus durans*, *O. niloticus* (Poot-Poot, 2001), *Streptococcus faecium* and *L. acidophilus* in *O. niloticus* (Lara-Flores et al., 2003), the commercial probiotic, Renascitur®, in *Ariopsis bonillai* (Rodríguez-Méndez et al., 2006), and *Bacillus amyloliquefaciens* (Mohammad et al., 2012).

Prebiotics are polysaccharides that stimulate growth and activity of one or several strains of bacteria in the host, improving health (Gibson and Roberfroid, 1995). Prebiotics include fructoligosaccharides, trans-galacto-oligosaccharides, mannanoligosaccharides, lactose, and inulin (Teitelbaum and Walker, 2002; Vulevic et al., 2004).

Inulin and its derivates (oligofructose and fructooligo-saccharides) are known as fructans and are constituted by linear fructose chains (Madrigal and Sangronis, 2007). Prebiotics have been used in fish like *Pagrus major* (Kihara et al., 1995), *Salvelinus alpinus* (Olsen et al., 2001), *Cyprinus carpio* (Kihara and Sakata, 2001a), *Onchorhynchus mykiss* (Kihara and Sakata, 2001b), *Psetta maxima* (Mahious et al., 2006), *Salmo salar* (Grisdale-Helland et al., 2008), *Huso huso* (Akramiet al., 2013), *Carassius auratus gibelio* (Xu et al., 2009), *Sciaenops ocellatus* (Zhou et al., 2010b) and *O. niloticus* (Ibrahem et al., 2010).

Fulvic acid is a derivative of humic acids, the latter being a complex mixture of organic material coming from leaves, branches, stems, etc., that decompose in soil. This decomposition process is accomplished by micro-organisms and fungi, producing fulvic acid and involves formation of compounds with very low molecular weight and positively charged ions (chelation process). Mineral chelated compounds are highly absorbable by plants and animals (Osman et al., 2009).

Fulvic acid increases growth and immune response in fish (Meinelt et al., 2001; Meinelt et al., 2002).

In this work, we isolated and characterized LAB from the intestine of tilapia and we determined their effect together with inulin and fulvic acid on growth in weight of *O. niloticus*, cultured under laboratory conditions.

MATERIALS AND METHODS

Isolation of presumptive LAB

Fish were collected (13.00 ± 12.84 g) from the Sinaloa River and from an agricultural irrigation channel (Guasave, Sinaloa, Mexico). Wild fish came from artificial dams. Petri plates were prepared with (Man, Rogosa and Sharp) (MRS) and Rogosa agar (BD Difco, Sparks, MD, USA). Each fish was measured, weighed, and disinfected with alcohol (70%). The whole intestine was extracted with a scalpel and macerated in a mortar with 150 µl of distilled water. From the macerated product, 100 µl were taken and transferred in the Rogosa agar plates using a triangular plastic loop. Petri plates were incubated at 30°C, for 48 h. The presumptive LAB colonies were identified (small, convex, circular, with smooth border, and white in color) and subsequently isolated again in MRS medium through cross streaking. Petri plates were incubated as mentioned before.

Isolated colonies were transferred on Petri plates with MRS medium and incubated at 30°C, for 48 h. Bacteria were harvested and re-suspended in Eppendorf tubes with 1 ml of MRS medium and 15% glycerol (v/v). The bacterial suspension was preserved at -80ºC.

Characterization of presumptive LAB

For the hemolysis test, Petri plates were prepared with blood agar with 5% human blood, making 6-mm diameter holes in the plate with a sterile holemaker. Isolates were cultured in MRS broth at 30°C, for 48 h, and centrifuged at 10,000 *g*, for 10 min. The LAB supernatant was adjusted to a pH of 6.5-7.0 with 1 M NaOH. Wells were inoculated with 50 µl of the supernatant and the plates were incubated at 30°C for 24 h. The lysis halo was observed to determine the type of hemolysis (alpha, beta, or gamma). Isolates with alpha or beta hemolysis were discarded because they can lysate cells.

Gram stain was performed only on isolates with gamma hemolysis (Golden Bell kit, Materiales y Abastos Especializados, S.A de C.V., Jalisco, Mexico).

Presumptive LAB isolates with gamma hemolysis were antagonized against *Vibrio* sp. They were transferred by distributing 5×10^4 CFU of vibrio in Petri dish containing trypticase soy agar (TS, BD Bioxon, Sparks, MD, USA), in which holes were made as mentioned before. Each well was inoculated with 50 µl of the supernatant from a 24 h liquid LAB and yeast cultures. The supernatant of yeast cultures was taken and the pH of the liquid LAB culture was adjusted as mentioned before. MRS broth was used as negative control. Plates were incubated for 24 h at 37°C, and the inhibition halo was measured (Bauer et al., 1966).

Growth kinetics (absorbance vs time) was applied to the selected LAB isolates (LAB 1-6, LAB 35, and LAB 37) to know the adaptation, exponential, and stationary stages. MRS broth (50 ml) was prepared and this was inoculated with 20 µl of the stock of each LAB isolate, and incubated at 30ºC. Absorbance (580 nm) was determined at 6, 12, 24, 48, 72, and 96 h in a spectro-photometer Thermo Spectronic Genesys 2 (Thermo Scientific, Rochester, NY, USA).

The hydrophobicity test was performed on the three selected isolates that were transferred through cross streaking in MRS agar medium supplemented with 0.03% Congo red (Sigma, St. Louis, MO, USA) and plates were incubated at 30°C, for 24-48 h. Positive results corresponded to those isolates with red coloring, and negative were those with white or translucent coloring (Sharma et al., 2006).

To obtain the LAB supernatant, the three selected isolates were cultured in MRS broth and incubated at 30°C, for 24-48 h. Samples were centrifuged at 10,000 *g* for 10 min to obtain the supernatant. The pH of the LAB supernatant was adjusted as mentioned above (León et al., 2000).

For the casein (proteases) degradation test, Petri plates were prepared with the basal medium (1.5% agar and 0.5% yeast extract) supplemented with 2% fat-reduced milk, once the medium had solidified, perforations were made as mentioned above. Each

hole was inoculated with 50 µl of the culture supernatant, using as control the culture medium. Plates were incubated at 30°C, for 24-48 h, considering those isolates with a transparent halo around the holeas positive.

For the gelatin hydrolysis test (proteases), Petri plates were prepared with basal medium supplemented with gelatin (1%). Perforations were made as mentioned above. Wells were inoculated with 50 µl of the culture supernatant, using as control the culture medium. Plates were incubated at 30°C, for 24-48 h, considering as positive result those isolates with an opaque halo around the well.

For the Tween 80 hydrolysis test (lipases), Petri plates were prepared with basal medium supplemented with 1% Tween 80 (Sigma, St. Louis, MO, USA), once the medium had solidified perforations were made as described above. Wells were inoculated with 50 µl of the culture supernatant, using as control the culture medium. Plates were incubated at 30°C, for 24-48 h, considering as positive result those isolates with an opaque halo around the well.

To count the selected LAB isolates, we performed a 24-h culture at 30ºC in MRS broth to count the CFU. Bacterial cultures were centrifuged at 10,000 *g* for 10 min, the supernatant was discarded and the pellet was re-suspended in 1 ml of sterile distilled water. The bacterial solution was adjusted to an optical density (580 nm) of 1, in a Thermo Spectronic Genesys 2 spectrophotometer. We determined the CFU/ml for each isolate, using the serial dilution method.

The DNA was extracted following the instructions of the Bactozol kit (MRC, Cincinnati, OH, USA). The quantification of DNA was performed with the Quant-iT™ dsDNA HS kit (Invitrogen, Carlsbad, CA, USA) and fluorescence was measured in a Qubit Q32854 equipment (Invitrogen).

For the molecular identification of the three LAB isolates with probiotic potential, we amplified genes 23S and 5S of the ribosomal DNA. Primers used for the PCR (P23S_F1 5'-GTTAAGTTATAAAGGGCGCATG-3' and P5S_R 5'-GCATGGCAACGTCCTAC-3') amplified a genome fragment of 3300 bp (Pfannebecker and Fröhlich, 2008). PCR conditions were those indicated by Pfannebecker and Fröhlich (2008). Cleaning of the PCR products was accomplished with the Wizard® SV Gel kit and PCR Clean-Up System (Promega, Madison, Wisconsin, USA), following manufacturer's instructions.

PCR products were sent to be sequenced (CINVESTAV, Irapuato, Mexico) with internal oligonucleotides designed by Leyva-Madrigal et al. (2011), which amplify a fragment of 550 bp.

Similarity searched was performed against the GenBank database of the National Center for Biotechnology Information (NCBI), using the BLAST program (Basic Local Alignment Search Tool). The phylogenetic analyses were performed with the Molecular Evolutionary Genetics Analysis software (MEGA 5 Beta) (Tamura et al., 2011). Evolutionary relationships among sequences were inferred by using the neighbor-joining method (NJ) (Saitou and Nei, 1987). The robustness of the NJ topology was evaluated by bootstrap test using 1000 replicates. The *Thermotoga maritima* sequence was used as outgroup.

Preparation of experimental diets with additives

Inulin, LAB, and fulvic acid were sprayed on commercial diet (Camaronina®, Purina, Mexico, 45% protein). Dry Oil® (Innovaciones Acuícolas, S.A. de C.V., Culiacán, Mexico) was used as adhesive and feed attractant. Diet was dried at room temperature overnight and stored at 4°C in a refrigerator for 10 days (Apún-Molina et al., 2009).

The LAB doses managed in the bioassay were based on the works of Apún-Molina et al. (2009) and Luna-González et al. (2013), whereas those of inulin were based on reports by Zhou et al. (2007) and Li et al. (2007) for *Litopenaeus vannamei*. The tested concentrations of fulvic acid were chosen empirically. The three selected isolates (LAB 1-6, LAB 35, and LAB 37) were mixed in the same proportions and then they were put in the diet.

Experimental design

Hormone-treated animals (males) weighing 1.3 ± 0.12 g were obtained from the farm DIBSA (Bacubirito, Sinaloa de Leyva, Sinaloa, Mexico) and acclimated for four days in two outdoor 1000-l plastic tanks with 800 l aerated fresh-water. To evaluate the effect of feed additives on growth performance of tilapia, the outdoor culture system was the same as for acclimation. The bioassay was conducted as a completely randomized design with four treatments in triplicate. The fish (12 organisms/tank) were fed *ad libitum* with floating pellets containing 45% protein. Water exchange (80%) and cleaning were performed weekly. Photoperiod was 12:12 h light: dark cycle. Values of temperature (HI 98127 pHep, Hanna Instruments, Woonsocket, RI, USA), pH (HI 98127 pHep, Hanna Instruments), and dissolved oxygen (YSI model 55 Oxygen meter, Yellow Spring Instruments, Yellow Springs, OH, USA) were determined weekly. Water samples for nitrite, nitrate, and ammonia determinations were analyzed at the beginning (first week of culture), middle, and end of each bioassay for all treatments following the Strickland and Parsons (1968) method. Growth in weight (g) was monitored twice a month by weighing each fish (12) of tanks in a balance (Scout Pro SP 601, Ohaus Corporation, Pine Brook, NJ, USA). Mortality was recorded daily.

The bioassay lasted 71 days: I) Control (commercial diet); II) LAB (5×10^4 CFU/g diet) + 2.5 g inulin + 2.5 g fulvic acid/kg diet; III) LAB (2.5×10^5 CFU/g diet) + 2.5 g inulin + 2.5 g fulvic acid/kg diet; IV) LAB (5×10^5 CFU/g diet) + 2.5 g inulin + 2.5 g fulvic acid/kg diet.

Specific growth rate

The specific growth rate (SGR) was calculated based on the following formula (Ziaei-Nejad et al., 2006):

$$SGR = 100 (\ln W - \ln W_0)/t$$

Where, W_0 represents the initial body weight of tilapia fry, W represents the final body weight of tilapia and t represents time in days.

Statistical analysis

A one-way analysis of variance (ANOVA) was used to examine growth differences among treatments. When significant differences were found, Tukey's HSD test was used to identify the source of these differences ($P < 0.05$).

RESULTS

From 37 presumptive LAB isolates, 26 presented beta-hemolysis and 11 presented gamma hemolysis. Gamma hemolysis is displayed by bacteria that do not induce hemolysis of the blood cells. Isolates with beta hemolysis were not characterized because of their capacity to lyse human and tilapia erythrocytes.

All presumptive LAB isolates were Gram (+), 11 were cocci, and four were bacilli. The cellular arrangement of LAB varied, some isolates presented an arrangement in pairs, others in chains, and others had a grape-like

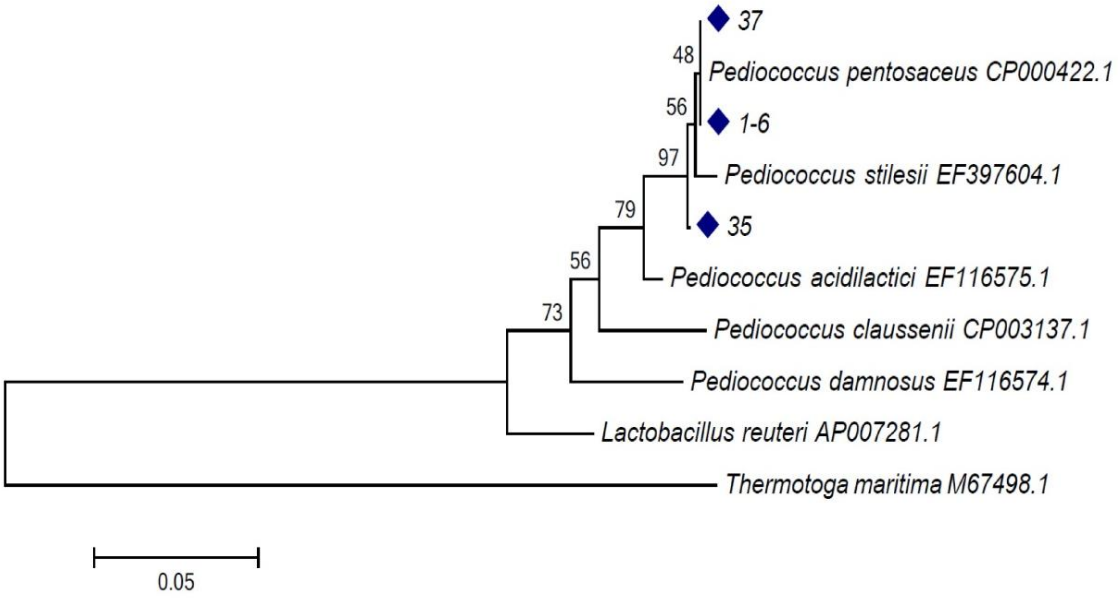

Figure 1. Phylogenetic tree (neighbor-joining) for LABs of *O. niloticus* and different LAB sequences (GenBank Access numbers are indicated) derived from partial sequences of the gene 23S rRNA. *Thermotoga maritima* was used as outgroup. Number on nodes indicates the bootstrap levels based on 1000 repeats. Bar = Sequence divergence.

arrangement. LAB isolates with the highest probiotic potential were chosen according to the following criteria: gamma hemolysis, hydrophobicity, Gram (+), and to be derived from fish that presented the largest number of bacteria in the intestine at the time of isolation.

The selected LAB isolates (LAB 1.6, LAB 35, and LAB 37) did not inhibit the *Vibrio* sp. isolate; however, LAB has other beneficial characteristics.

Isolate LAB 35 had an exponential phase of approximately 66 h and for the rest it was of 46 to 48 h. Microorganisms are applied to the diet when the culture is in its exponential phase.

None of the selected isolates presented extracellular enzymatic activity (proteases and lipases); however, all LAB isolates were hydrophobic, which is an important feature as it indicates that the isolates had the capacity to adhere to the tilapia's intestine.

The number of CFU/ml of LAB ranged between 17.2 and 24.7 millions. This value served to add the microorganisms to the diet in adequate amounts.

The isolates LAB 1-6 and LAB 37 depicted 100% similitude (genetic similarity) with *P. pentosaceus*, reported in the GenBank database, and group together to form a monophyletic group. The isolate LAB 35 depicted 99.6% similitude with *P. pentosaceus* but did not form a monophyletic group. The isolate LAB 35 was identified only at genus (*Pediococcus*) level (Figure 1).

The physicochemical water parameters, recorded during the study, were within the recommended tolerance range for Nile tilapia (Jiazhao, 1991; Popma and Lovshin, 1996) with exception of ammonia (0.30 ± 0.3 - 0.481 ± 0.4 mg/l) that was above the critical value (0.1). The final survival was 100% in all treatments. Fish fed with LAB (2.5×10^5 CFU/g of diet) plus inulin and fulvic acid (treatment III) grew significantly ($P = 0.03$) better than the control (Figure 2). Results demonstrate that additives influenced positively the growth of organisms in the concentrations of the treatment III.

DISCUSSION

The use of probiotics in aquaculture has intensified in the last years (Verschuere et al., 2000; Balcazar et al., 2008). To select the microorganisms with probiotic potential, these must be isolated preferably from the same organism in which they are going to be tested and should be from the same region (Verschuere et al., 2000), since there are many commercial probiotics that are less suitable because they were isolated in other regions or countries. In this work, we obtained 37 presumptive LAB isolates from the intestine of *O. niloticus* for their characterrization as potential probiotics; the same species.

A first criterion to select isolates with probiotic potential was the analysis of hemolysis as a virulence factor, since many organisms are able to synthesize exotoxins that induce partial or total lysis of human or animal erythrocytes (Gildberg et al., 1995; Zamora-Rodríguez, 2003). In this study, LAB with alpha or beta hemolysis (hemolytic activity) was discarded and only those with gamma hemolysis (lacking hemolytic activity) were selected.

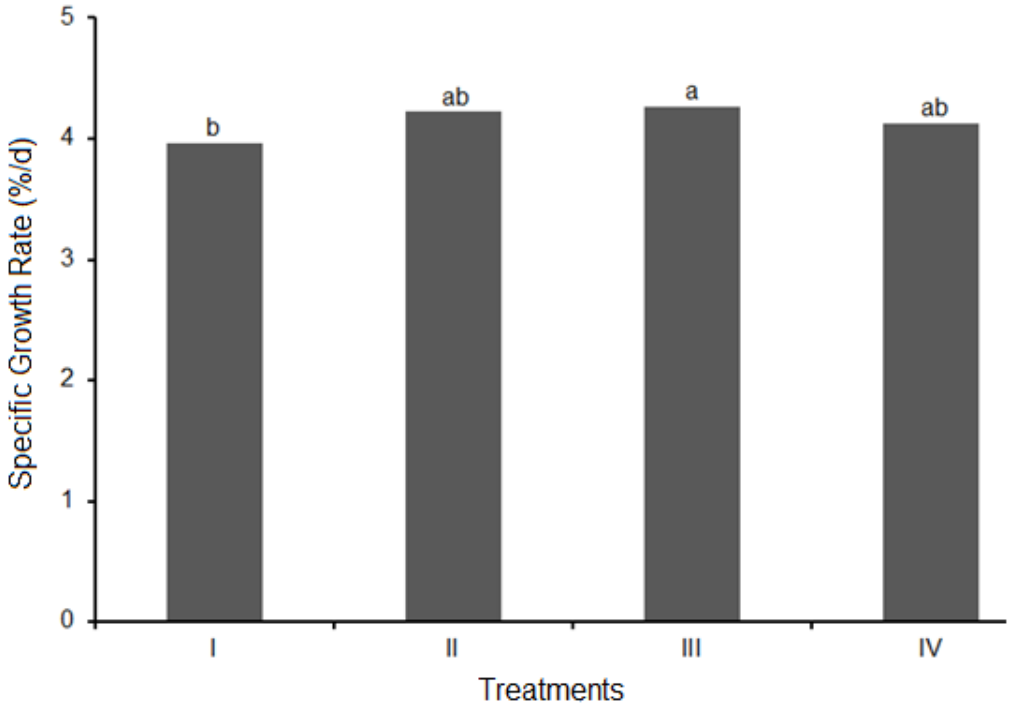

Figure 2. Specific growth rate of *O. niloticus*. Treatments: I) Control (commercial diet); II) LAB (5×10^4 CFU/g of diet) + 2.5 g inulin + 2.5 g fulvic acid/kg diet; III) LAB (2.5×10^5 CFU/g diet) + 2.5 g de inulin + 2.5 g fulvic acid/kg diet; IV) LAB (5×10^5 CFU/g of diet) + 2.5 g de inulin + 2.5 g fulvic acid/kg diet. Data are expressed as average ± SD (standard deviation). Different letters indicate significant differences ($P < 0.05$).

The presumptive LAB isolates with gamma hemolysis were Gram (+), mostly cocci, and very few were bacilli; their arrangement was in pairs, some in chains and others presented a grape-like arrangement. According to Axelsson (1998), in general, LABs can be characterized as Gram (+), anaerobic or facultative anaerobes, and rods or cocci that do not form spores. Besides they are oxidase negative, catalase negative, and benzidine-negative, lack cytochromes, do not reduce nitrites to nitrates, are gelatinase-negative, and are unable to use lactate.

The three selected LAB isolates with the best probiotic potential did not antagonize the used *Vibrio* isolate, which disagrees with one of the characteristics recommended for the selection of probiotics, that is competitive exclusion of noxious bacteria due to antibiotic production (bacteriocins, lysozymes, proteases, and H_2O_2) (Bruno and Montville, 1993; Naidu et al.,1999; Bjorn et al., 2003). However, the probiotic effect can be provided by the pH decrease due to the production of lactic acid, acetic acid (Aguirre-Gúzmán, 1994), fatty acids, and other molecules (Midolo et al., 1995; Kao and Frazier, 1996). In addition, according to Bruno and Montville (1993), it is assumed that LAB could have other action mechanisms, such as improving water quality by degrading organic matter.

Establishing growth kinetics of the isolates is considered useful since it allows for strategies for the manipulation. It is important to know the exponential phase of the isolates because if Gram stain is performed on a bacterium in stationary phase or declining stage an erroneous result can be obtained (Prescott et al., 1999). This is because old cultures can lose their ability to retain the crystal violet complex characteristic of Gram (+) staining.

The criteria to select bacteria with probiotic potential vary depending on the study. Some authors state that the extracellular production of enzymes like proteases and lipases provide positive additives to the nutrition of the host (Moriarty, 1999; Balcazar et al., 2008), whereas other authors consider that the excessive production of these enzymes is a pathogenicity factor because pathogenic strains depict a high extracellular proteolytic, lipolytic, and hemolytic activity (Quesada-Herrera et al., 2004). Therefore, we performed the tests to determine these activities. However, none of the selected LAB presented proteases and lipases activity.

The three LAB isolates showed a positive hydrophobicity, which means that the organisms are able to bind non-specifically to the intestinal epithelium through hydrophobic interactions. According to Rinkinen (2004), if these hydrophobic molecules did not exist on the surface of microorganisms, they could not attach to the epithelium, since both are negatively charged. However, it is important to mention that the hydrophobic interactions also favor adhesion and colonization of some pathogens.

Molecular identification of the three LAB isolates revealed 99.6 to 100% identity with *P. pentosaceus*, which is a species generally inhabiting vegetal material, cheese, and meat; however, its isolation has been reported from the intestines of tiger shrimp (*Penaeus monodon*), brown shrimp (*Farfantepenaeus californiensis*), damselfish (*Dascyllus aruanus*), cinnabar goatfish (*Parupeneus cinnabarinus*), sand shrimp (*Metapenaeus ensis*), green mussel (*Perna viridis*), and white shrimp (*L. vannamei*), among other organisms (Buntin et al., 2008; Leyva-Madrigal et al., 2011).

The bioassay showed significant differences in growth between treatment III and the control group. There are no reports about the effect of LAB, inulin, and fulvic acid on growth of *O. niloticus*. However, in terms of probiotics, our results agree with those of Apún-Molina et al. (2009), who added lactococci to the diet, achieving a better growth than in the control without bacteria and similar survivals to those found in the control without probiotics. Likewise, a positive effect on growth was found in *O. niloticus* fed with diets supplemented with *Lactobacillus acidophillus*, *Saccharomyces cerevisiae*, *Streptococcus faecium*, *Bacillus* sp., and *Lactobacillus* sp. (Lara-Flores et al., 2003). Also, a positive effect on growth was observed in tilapia fed with a diet supplemented with *Bacillus* sp. and *Lactobacillus* sp. (El-Haroun et al., 2006) or with *Bacillus* sp., *Lactobacillus* sp., and *Saccharomyces* sp. (Guevara et al., 2003).

Characterization and selection of isolates was adequate and the combination of *Pediococcus* sp., *P. pentosaceus*, fulvic acid, and inulin can be added to the diet to improve growth of *O. niloticus*. However, it is necessary to investigate the effect of the tested additives on the immune system of *O. niloticus*.

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Full Length Research Paper

Molecular epidemiological study and detection of multi-drug resistant *Acinetobacter baumannii* -related resistance genes

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This study aimed to investigate the existence and molecular epidemiological characteristics of multi-drug resistant *Acinetobacter baumannii* (MDRAB)-related resistance genes isolated from our hospital. Microdilution and disk diffusion methods were used to detect the antibiotic susceptibility of 46 MDRAB isolates that carried OXA-51 gene. Pulsed-field gel electrophoresis (PFGE) was used to analyze the homologies among the strains. Resistance genes were tested through polymerase chain reaction. Among the 46 MDRABA, a total of nine, isolates were categorized as strain A (19.6%), eight as strain B (17.4%), and four as strain O (8.7%). A total of 41 isolates (89.1%) carried the OXA23 gene, 17 (37.0%) carried the PER gene, and six (13%) carried the IMP gene. Six strains (13%) did not carry the membrane pore protein gene carO. The numbers of isolates that carried a particular gene were as follows: 40 (87.0%), armA; 41 (89.1%), ant(3")-I; 33 (71.7%), aac(3)-I; 2 (4.3%), aac(3)-II; 1 (2.2%), aac(6')-II; and 1 (2.2%) and aph(3')-VI. Most of the isolates (93.5%) carried the qacE△1 gene. These hospital MDRAB isolates were proven to simultaneously carry a variety of drug resistance genes. Strains A and B were the major epidemic strains of nosocomial MDRAB.

Key words: *Acinetobacter baumannii*, resistance gene, pulsed-field gel electrophoresis, multi-drug resistant.

INTRODUCTION

Acinetobacter baumannii has recently emerged as a significant pathogen, with a surprisingly rapid acquisition of antibiotic resistance and fast proliferation within hospitals (Jiang et al., 2012). Several studies have demonstrated that Multidrug-resistant *A. baumannii* (MDRAB) and extensive drug-resistant *A. baumannii* have caused significant challenges in clinical treatment (Munoz and Weinstein, 2008; Peleg et al., 2008; Doi et al., 2009). Carbapenems, fluoroquinolones, aminoglycosides, sulbactam, and sulbatacm combinations are often used against *A. baumannii*. However, the number and incidence of MDRAB strains had increased considerably during the past decade. The current study examined the β-lactam, aminoglycoside, fluoroquinolones, and disinfectant-related resistance genes (qacE△1 gene) of MDRAB strains isolated from our hospital. In addition, the statistical distribution of the resistant strains was also investigated to analyze the resistance mechanisms and relationships among MDRAB strains from our hospital.

MATERIALS AND METHODS

Source of strain

Clinical isolates were isolated from the Microbiology Laboratory in our hospital using standard procedures. A total of 46 MDRAB isolates were collected from June 2010 to June 2011, of which 44 isolates were from the sputum, one from puncture fluid, and one

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from secretions.

Bacterial identification and antibiotic susceptibility determination

MDRAB is *A. baumannii* resistant to multiple antibiotics, often defined as three or more antimicrobials (aminoglycoside, ampicillin-sulbactam, antipseudomonal carbapenem, antipseudomonal cephalosporin, fluoroquinolone). The bacteria were identified using WalkAway 96 PLUS NC31 composite panels. Antimicrobial susceptibility test was performed using the Kirby-Bauer disk diffusion method. Susceptibility testing was performed in accordance with Clinical and Laboratory Standards Institute Guidelines of 2011. Susceptibility was tested for the following antimicrobial agents: amikacin, ceftazidime, ceftriaxone, ciprofloxacin, cefepime, gentamicin, imipenem, levofloxacin, meropenem, piperacilin, cotrimoxazole, piperacilkin/tazobactam, tobramycin, cefoperazone / sulbactam, minocycline, and tigecycline. Susceptibility to polymyxin E was examined using ATB PSE strips. MH agar and susceptibility paper were both purchased from British Oxoid.

Resistance gene detection

Bacterial DNA was extracted using the boil extraction method and resistance gene detection was performed via polymerase chain reaction (PCR). Table 1 lists the pairs of oligonucleotide primers designed to target β-lactam resistance genes. The primers were designed according to previously published procedure (Shen et al., 2008; Hu et al., 2008). Sixteen (16) pairs of oligonucleotide primers (Table 2) were designed to target aminoglycosides, plasmid-mediated fluoroquinolones, and disinfectant-related resistance genes. The primers were designed according to previously published procedure (Zhi et al., 2005; Wang et al., 2009; Yang et al., 2011; Chi et al., 2006; Turton et al., 2006).

A number of positive gene PCR products were analyzed for nucleotide sequence by Shanghai Sunny Biotechnology Co., Ltd. The resulting sequence data were compared with the data from the GenBank database using BLAST at the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov/).

Homology analysis

Pulsed-field gel electrophoresis (PFGE) was performed following the protocol of Gautom with some modifications. The PFGE patterns were analyzed by computer-assisted program (Bio-Numerics software. Version 5.1, Appliedmaths, Inc.) and by manual or visual comparison of each band patterns. Band patterns were compared and classified as nondistinguishable and unrelated with 90% as the homology threshold.

RESULT

Antibiotic Resistance

The sensitivities of the 46 strains to 17 antimicrobials are presented in Table 3.

PFGE

The PFGE patterns of the 46 MDRAB isolates were analyzed using computer-assisted program (BioNumerics software. Version 5.1, Appliedmaths, Inc.) and by manual or visual comparison of each band patterns. A 90% interlinkage homology level between patterns was considered as the cutoff for defining close genetic relationship isolates. Isolates were categorized into 20 major groups and designated as Strains A to T based on the generated dendrogram (Figure 1). A total of nine isolates were categorized as, strain A (19.6%), eight as strain B (17.4%), and four as strain O (8.7%). The other types of strains only contained 1 or 2 isolates. All strains were found in the intensive care wards. The distribution of which is shown in Table 4.

Detection of β-lactam resistance gene and sequencing results

Approximately 100% of the 46 MDRAB isolates carried β-lactam resistance genes. The numbers of isolates that carried a particular gene were as follows: 41 (89.1%) OXA23, 17 (37%) PER, 6 (13%) IMP, and 4 (8.7%) of both OXA23 and IMP-4 genes. Sequence analysis of PCR amplification products of the three OXA23 gene-positive isolates showed 100% homology with JN207493. Four of the amplification products of the six IMPgene positive isolate showed that four isolates were 100% homologous to JN106667 and the rest only showed 99%. The sequence analysis of the PCR amplification products of 4 PER gene-positive isolates showed 99% homology with FE535600.

Detections of aminoglycoside and plasmid-mediated quinolone resistance genes as well as qacEΔ1 gene

The numbers of isolates among the 46 MDRAB isolates that carried a particular gene were as follows: 40 (87.0%), armA; 41 (89.1%), ant(3")-; 33 (71.7%), aac(3)-I; 2 (4.3%), aac(3)-II; 1 (2.2%), aac(6')-II; 1 (2.2%), aph(3')-VI; 41 (89.1%), AMES; 40 (87%), 16SrRNA methylase; and 43 (93.5%), qacEΔ1. The aac(3)-III, aac(3)-IV, aac(6')-I, and ant(2")-I AMES genes were not detected. No rmtB16SrRNA methylase gene was detected. Plasmid-mediated fluoroquinolone resistance genes, namely, qnrA, qnrB, or qnrC, were not detected. Three MDRAB isolates that only carried armA16SrRNA methylase genes were resistant to amikacin, tobramycin, and gentamicin. Two isolates that did not carry AMES or 16SrRNA methylase were sensitive to amikacin, tobramycin, and gentamicin.

Analysis of the clinical data

A total of 44 isolates collected from sputum samples of patients carried MDRAB. These patients had symptoms of respiratory tract infection. MDRAB were detected in both sputum samples and thoracentesis fluid from 1 patient. MDRAB was detected in the fluid drained from one patient' after surgery. All patients with MDRAB

Table 1. PCR ampification primer sequences.

Primer	Sequence of primers (5'→3')	Size (bp)
OXA23gp-F	CCCCGAGTCAGATTGTTC	291
OXA23gp-R	GCTTCATGGCTTCTCCTAG	
OXA24gp-F	ACGAGCAAATAAAGAATATGTCCC	496
OXA24gp-R	CACCCAACCAGTCAACCAAC	
OXA48gp-F	GGGATGGACAGACMCGSGATA	300
OXA48gp-R	TGGCTTGRTTGACYATACGC	
OXA55gp-F	GCTGAGGGTTGGCAAGAGGT	179
OXA55gp-R	AACGCAATAAGGCTGGAGGG	
OXA58gp-F	TGGCACGCATTTAGACCG	507
OXA58gp-R	AAACCCACATACCAACCC	
OXA60gp-F	TCACCGCCGACCGTACCTAT	177
OXA60gp-R	CGTGCTCCCACTGCTCGTAA	
OXA64gp-F	TCAGCCTGCTCACCTTAT	406
OXA64gp-R	CACGCTTCACTTCKTTAGAC	
OXA66qc-F	ATGAACATTAAAGCACTC	825
OXA66qc-R	CTATAAAATACCTAATTG	
VIM-2gp	TCCGACAGTCAYCGAAAT	435
VIM-2gp	GCAGCACCYGGATAGAAGAG	
VIM-7	TACACCTCACCCTTGACACGC	246
VIM-7	ATTGGCATCGGCAACATTAC	
IMP-1gp-F	TCTCATTTTCATAGRGACAG	353
IMP-1gp-R	ACCAGTTTTGCCTTACCATA	
IMP-2gp-F	CTTGTAAACACWGACGCCTAT	134
IMP-2gp-R	GTGCTGTCGCTATGGAAAT	
IMP-11gp-F	GKGTCTTTGCCTGATTTA	256
IMP-11gp-R	CTATTCCACCCGWGCTGT	
IMP-14gp-F	RGACAGTACGGCTGGAATAG	239
IMP-14gp-R	CAAAGCAACCACCGAATAAA	
IMP-12gp-F	TTRCATAGCGACAGRACG	277
IMP-12gp-R	CARCCAAATTACCWAGACC	
GIM1-F	ATTACTTGTAGCGTTGCC	418
GIM1-R	CTCTATAAGCCCATTTCC	
SPM1-F	GCCATCAATACGCACTTTCA	526
SPM1-R	ACAGTCTCATTTCGCCAACG	
KPC-gp-F	GCGGAACCATTCGCTAAACTC	340
KPC-gp-R	CGCCCAACTCCTTCAGCAACA	
TEM-F	AGGAAGAGTATGATTCAACA	535
TEM-R	CTCGTCGTTTGGTATGGC	
SHV-F	GGTTATGCGTTATATTCGCC	867
SHV-R	TTAGCGTTGCCAGTGCTC	
PER-F	AGTCAGCGGCTTAGATA	978
PER-R	CGTATGAAAAGGACAATC	
EBC-MF	TCGGTAAAGCCGATGTTGCGG	302
EBC-MR	CTTCCACTGCGGCTGCCAGTT	
VEB-F	GCGGTAATTTAACCAGA	961
VEB-R	GCCTATGAGCCAGTGTT	
MOX-F	GCTGCTCAAGGAGCACAGGAT	520
MOX-F	CACATTGACATAGGTGTGGTGC	
CTT-MF	TGGCCAGAACTGACAGGCAAA	462
CTT-MR	TTTCTCCTGAACGTGGCTGGC	
ACC- MF	AACAGCCTCAGCAGCCGGTTA	346

Table 1. Contd.

ACC- MR	TTCGCCGCAATCATCCCTAGC	
FOX- MF	AACATGGGGTATCAGGGAGATG	190
FOX- MR	CAAAGCGCGTAACCGGATTGG	

Table 2. PCR ampification primer sequences.

Target gene	Sequence of primers(5'→3')	Size (bp)
aac(3)-Ⅰ	P1:ACCTACTCCCAACATCAGCC P2:ATATAGATCTCACTACGCGC	169
aac(3)-Ⅱ	P1:ACTGTGATGGGATACGCGTC P2:CTCCGTCAGCGTTTCAGCTA	237
aac(3)-Ⅲ	P1: CACAAGAACGTGGTCCGCTA P2:AACAGGTAAGCATCCGCATC	185
aac(3)-Ⅳ	P1:CTTCAGGATGGCAAGTTGGT P2:TCATCTCGTTCTCCGCTCAT	286
aac(6′)-Ⅰ	P1:TATGAGTGGCTAAATCGA P2:CCCGCTTTCTCGTAGCA	394
aac(6′)-Ⅱ	P1: TTCATGTCCGCGAGCACCCC P2:GACTCTTCCGCCATCGCTCT	178
aph(3′)-Ⅵ	P1:ATACAGAGACCACCATACAGT P2:GGACAATCAATAATAGCAAT	234
ant(3″)-Ⅰ	P1:TGATTTGCTGGTTACGGTGAC P2:CGCTATGTTCTCTTGCTTTTG	284
ant(2″)-Ⅰ	P1:GAGCGAAATCTGCCGCTCTGG P2:CTGTTACAACGGACTGGCCGC	320
armA	P1:AGGTTGTTTCCATTTCTGAG P2:TCTCTTCCATTCCCTTCTCC	591
rmtB	P1:ATGAACATCAACGATGCCC P2:CCTTCTGATTGGCTTATCCA	769
qnrA	P1:ATTTCTCACGCCAGGATTTG P2:GATCGGCAAAGGTTAGGTCA	516
qnrB	P1:GATCGTGAAAGCCAGAAAGG P2: ACGATGCCTGGTAGTTGTCC	469
qnrS	P1:ACGACATTCGTCAACTGCAA P2: TAAATTGGCACCCTGTAGGC	417
aac（6’）-Ib	P1:TTGCGATGCTCTATGAGTGGCTA P2: CTCGAATGCCTGGCGTGTTT	482
qacE△1	TAGCGAGGGCTTTACCTAAGC ATTCAGAATGCCGAACACCG	300

Table 3. Susceptibility results of 46 isolates of MDRAB.

Antimicrobial drugs	Sensitive (number (%) of isolates)	Intermediary resistance (number (%) of isolates)	Resistance (number (%) of isolates)
Amikacin	3(6.5)	0(0.0)	43(93.5)
Ceftazidime	0(0.0)	0(0.0)	46(100)
Ceftriaxone	0(0.0)	0(0.0)	46(100)
Ciprofloxacin	0(0.0)	0 (0.0)	46(100)
Cefepime	0(0.0)	0(0.0)	46(100)

Table 3. Contd

Gentamicin	2(4.3)	1(2.2)	43(93.5)
Imipenem	0(0.0)	0 (0.0)	46(100)
Levofloxacin	0(0.0)	2(4.3)	44(95.7)
Meropenem	0(0.0)	0(0.0)	46(100)
Piperacilin	0(0.0)	0(0.0)	46(100)
Cotrimoxazole	1(2.2)	0(0.0)	45(97.8)
Piperacillin/tazobactam	0(0.0)	0(0.0)	46(100)
Tobramycin	3(6.5)	0(0.0)	43(93.5)
Cefoperazone/sulbactam	6(14)	15(32)	25(54)
Minocycline	25(54.3)	20(43.5)	1(2.2)
Tigecycline	28(60.9)	0(0.0)	
Polymyxin E	46(100)		

Table 4. Distribution of 20 strains in the intensive care wards.

ICU	Number of strain																			
	A	B	C	D	E	F	G	H	Y	Z	K	L	M	N	O	P	Q	R	S	T
ICU1	5	0	0	0	1	0	1	0	2	1	1	0	0	0	1	2	1	1	0	0
ICU2	2	1	1	1	0	0	0	2	0	1	0	0	1	0	2	0	0	0	0	0
ICU3	1	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	1	1
ICU4	0	7	0	1	0	1	0	0	0	0	0	2	0	0	0	0	0	0	0	0
ICU5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ICU6	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0

(46/46) used broad-spectrum antimicrobial drugs and suffered from a variety of acute and (or) chronic diseases.

DISCUSSION

MDRAB has emerged as the most severe nosocomial pathogens . A previous study has reported that suction devicesand ventilator, as well as air conditioning, andtransfusion systems can be contaminated with this pathogen, spreading, MDRAB to patients (Shen and Ou, 2008). *A. baumannii* is the most common opportunistic pathogen that causes respiratory tract infection. Among the 46 MDRAB isolates from our hospital, 44 isolates were collected from sputum samples of patients carrying MSRAB. All of the 44 patients exhibited symptoms of respiratory tract infection.

Carbapenemases are β-lactamases with various hydrolytic capacities. Carbapenemases are members of molecular classes A, B, and D β-lactamases. KPC carbapenemases are the most prevalent in the class A carapenemase group. Class B enzymes include metallo-β-lactamases which contain zinc in the active site and belong to the IMP, VIM, SPM, CIM, and SIM families. These enzymes have been detected primarily in *Pseudomonas aeruginosa. The class D carbapenemases consist of* OXA-type β-lactamases. OXA-23, which can hydrolyze carbopenems antibiotics, is one of main factors that caused the multi-drug resistance of *A. baumannii* (Zou et al., 2010; Yu et al., 2011). The OXA enzyme is the major product of *A. baumannii*. Several strains of *A. baumannii* also produce extended-spectrum β-lactamase, cephalosporinase, and metal enzyme, which can result in resistance to β-lactam antimicrobial agents. For example, IMP-4 mental enzymecan hydrolyze β-lactam antibiotics, including carbopenem and cephalosporin antibiotics. In addition, PRE-1 Extended-spectrum β-lactam enzyme can hydrolyze cephalosporin antibiotics. Our study shows that 41 isolates carried the OXA23gene (89.1%), 17 (37%)) the PER gene, and 6 (13%) carried the IMP gene. The sequence analysis of the PCR amplification products of three OXA23 gene positive isolates showed that they were all OXA-23 gene. The sequence analysis of the PCR amplification products of four PER gene positive isolates showed that they were all PER-1 gene. The results showed that OXA-23 is the most common carbapenemases gene among the MDRAB isolates collected from our hospital. This phenomenon is similar to the results of Ma et al. (2011) in which the OXA-type enzyme carbapenemase was found to have an important function in imipenem resistance of *A. baumannii* and the OXA-23 type enzyme is common in China. In addition, PER-1 and

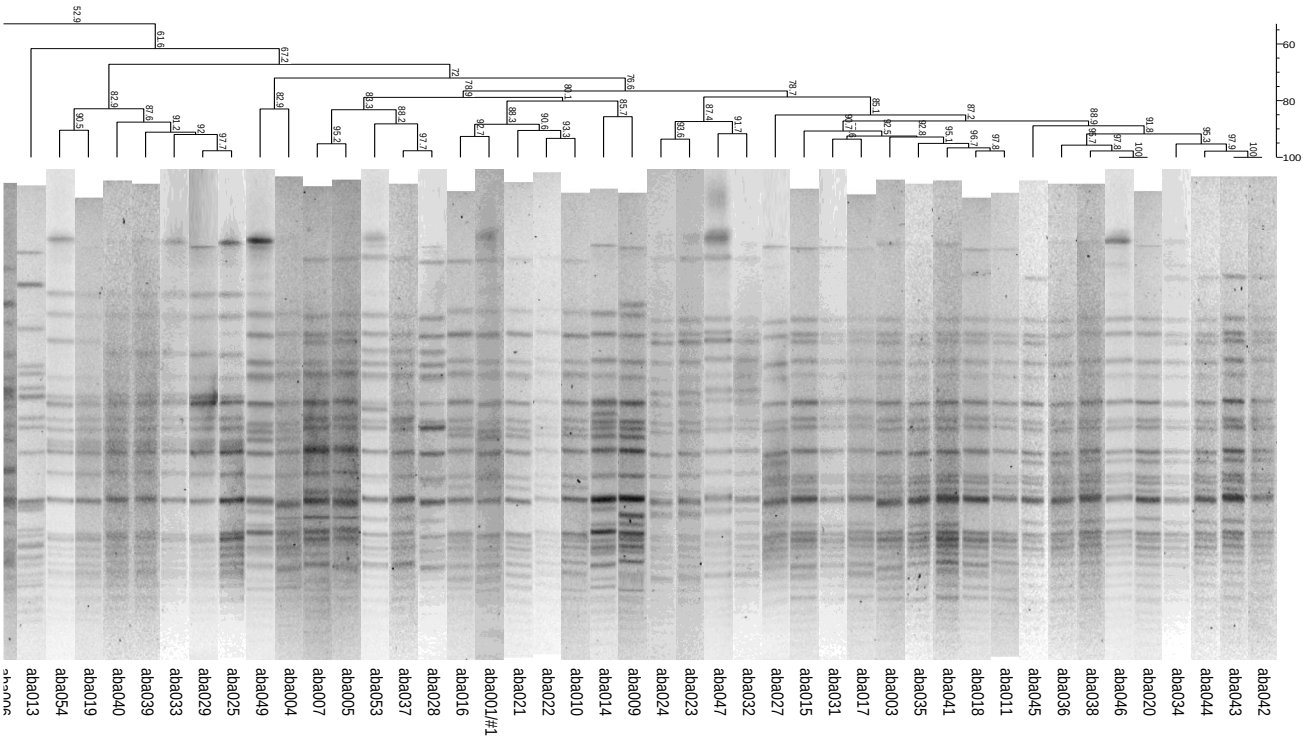

Figure 1. PFGE results.

IMP-4 mental enzymes may be related to the β-lactam antibiotic resistance of MDRAB in our hospital.

Galimand et al. (2000) reported that resistance to aminoglycoside antibiotics was caused by actions of aminoglycoside-modifying enzyme (AME) and mutations in the 16SrRNA genes of aminoglycoside antibiotics'. AMEs can be divided into acetyltransferase, phosphotransferase, and nucleoside transferase according to the different functions of the enzymes. More than 30 AME enzymes had been found (Chang et al., 2004). A total of 46 MDRAB isolates were found in our study, of which 41 isolates carried ant (of which enaac(3)-I gene, 2 carried aac(3)-II gene, 1 carried aac(6c(6I gene, 1 carried aac (6aph(3h(3I gene, 1 carried aac(6c(6 gene, 1 was 89.1%, indicating that the resistance of MDRAB in our hospital to animoglycoside drug is related with AMEs. The positive rate of armA 16SrRNA was 87% (40/46), which is higher than the positive rate of 69.77% in 43 isolates of *A. baumannii* resistant to amilacin reported by Miao et al. (2011). Feng et al. (2008) have reported that the 16S rRNA methylase *armA* and *rmtB* genes were not detected in 20 MDRAB isolated. 16S rRNA methylase may have an important role in the resistance of MDRAB to aminoglycoside antibiotics in our hospital. In the current study, three MDRAB isolates that only carry the armA 16SrRNA methylase gene were resistant to amikacin, tobramycin, and gentamicin. Thus, armA 16SrRNA methylase gene may lead to resistance to aminoglycoside antibiotics alone. One isolate of MDRAB that only carry AMEs was sensitive to amikacin and tobramycin and was intermediary resistant to gentamicin. Two MDRAB isolates without AMEs or 16SrRNA methylase gene were all sensitive to amikacin, tobramycin, and gentamicin. These results indicate that AMEs and 16SrRNA methylase gene were related with the resistance of MDRAB' to aminoglycoside antibiotics. The positive rate of qacEΔ1 in our hospital was 93.5% (43/46). The high resistance to disinfectants may be an important factor of nosocomial infection, which should be addressed by the disinfection department in our hospital.

The existence of plasmid-mediated fluoroquinolone resistance gene in MDRAB has been reported in several cities in China. The qnr gene positive rate was 43.9% in 57 isolates of *Klebsiella pneumoniae* producing extended - spectrum β-lactamase. Although the qnr gene was mainly detected from Enterobacter sp, Yang et al. (2009) reported that 2 of the 115 *A. baumannii* isolates were qnrB gene-positive. No plasmid-mediated quinolone fluoroquinolone resistance gene was detected from the 46 MDRAB isolates in our hospital, so MDRAB resistance to fluoroquinolones was not related to the plasmid-mediated fluoroquinolone resistance gene.

Among the 46 patients with MDRAB, 45 patients had symptoms of respiratory tract infection. The 46 patients used broad-spectrum antimicrobial drugs and were suffering from a variety of acute and (or) chronic diseases. Among the nine patients infected by MDRAB strain A, four patients were cured, three patients improved, and

two patients died. Analytical results show that several infected patients were related to nosocomial infections. However, the clinical effect was related to the patients' physical condition and the proper use of antibacterial drugs. A total of 44 isolates were collected from the sputum samples of patients with MDRAB. Although they were all qualified specimens, distinguishing infectious bacteria from bacterial colonization was difficult. Therefore, clinicians should improve the submission rate of blood culture.

Table 4 shows that nine isolates of MDRAB strain A distributed in four intensive care units in our hospital were mainly derived from ICU 1. Eight isolates of MDRAB strain B were distributed in two intensive care units, seven came from ICU 2, and four isolates of MDRAB strain O were distributed in three intensive care units. Therefore, the common MDRAB stains in our hospital were mainly strains A and B, followed by strain O. Some of the patients infected by MDRAB strain A or B were transferred from other hospitals, so strains A and B may be the major epidemic MDRAB strains in this area.

In our study, the resistance rate of MDRAB to gentamicin, tobramycin, and amilacin were 93%. Two isolates exhibited intermediary resistance to levofloxacin. All the isolates were resistant to ciprofloxacin, ceftazidime, ceftriaxone, ciprofloxacin, cefepime, imipene, meropenem, piperacillin, and piperacillin/tazobactam. They also exhibited high sensitivity to tigecycline (60.9%) and minocycline (54.3%). The resistance rate to cefoperazone/sulbactam was 54%, and the intermediary resistance rates to cefoperazone/sulbactam were 32%. One of the isolates was sensitive to cotrimoxazole. None of the isolates was resistant to polymyxin E. Therefore, treatments of choice for MDRAB infection in our hospital are polymyxin + cefoperazone/sulbactam; and cefoperazone/sulbactam + minocycline or cefoperazone / sulbactam+ tigecycline.

Attention should be paid when clinicians select treatments for MDRAB infections because optional antibiotics for MDRAB are very limited. Clinicians should be aware of the characteristics of MDRAB in the area and strengthen the awareness of drug combination. Moreover, rational use of drug and prevention of the spread of resistant bacterium are important factors for controlling MDRAB in our hospital.

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Full Length Research Paper

Diversity and abundance of arbuscular mycorrhizal fungi associated with acacia trees from different land use systems in Ethiopia

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Root samples and rhizosphere soil of nine acacia species (*Acacia abyssinica*, *Faidherbia albida*, *A. nilotica*, *A. senegal*, *A. seyal*, *A. sieberiana*, *A. saligna*, *A. tortilis* and *A. robusta*) were collected from Bishoftu, Zeway and Addis Ababa sites with different land use types to assess their Arbuscular Mycorrhizal Fungal (AMF) diversity, spore density and root colonization. The percentage of root length colonized by AMF was estimated. Spores, spore clusters and sporocarps extracted from soil samples were counted and morphologically identified to species or specific morphotype. Roots of all acacia species were colonized from low to moderate or relatively high levels by AMF with the occurrence of arbuscules, vesicles and hyphae. Arbuscules were however not detected in roots of *A. senegal*. The highest AM fungal colonization was found in *A. seyal* (67.3%) from open grazing field (OGF) at Zeway followed by *A. nilotica* (44%), whereas the lowest AMF colonization of 12% was recorded in *A. saligna* at Bishoftu. Rhizosphere soils harbored AMF fungal spores ranging from 3.7 spores g^{-1} soil in *A. nilotica* to 15.0 spores g^{-1} in *A. seyal* from open grazing field (OGF) at Zeway. A total of 41 AMF species in 14 genera and 7 families of the Glomeromycota were identified. Nine species belonged to *Acaulospora*, 6 to *Funneliformis*, 4 each to *Gigaspora*, *Glomus*, and *Rhizophagus*, 3 each to *Claroideoglomus*, and *Scutellospora*, 2 each to *Racocetra* and *Diversispora*, and 1 each to *Entrophospora*, *Sclerocystis*, *Paraglomus* and *Pacispora.* Moreover, 2 unidentified morphotypes each of *Glomus*, and *Acaulospora* and 1 of *Archaeospora* were isolated. Based on relative abundance and isolation frequency of spores, *C. claroideum*, *C. etunicatum*, *C. luteum*, *F. geosporus* and *G. aggregatum* were the dominant species in the study. The study showed that the acacia species were characterized by relatively high AMF colonization and very high AMF diversity. AMF spore density and AM root colonization in acacia roots were influenced by soil factors such as available P and soil texture.

Key words: *Acaulospora*, AM colonization, Arbuscules, *Funneliformis*, Glomeromycota, Rhizosphere soils.

INTRODUCTION

The acacia trees are important legumes in the tropics represented by more than 1200 species (Anon, 1983). They are multi-purpose and fast growing woody plants used as fuel wood, fodder, for improving soil fertility and as shade for planting crops (Brewbaker, 1986). *Acacia* species with few exceptions nodulate and fix nitrogen with root nodule bacteria to the tune of 20 to 300 kg $ha^{-1}yr^{-1}$ (Dommergues, 1987), and symbiotically associated with Arbuscular Mycorrhizal Fungi (AMF) which is a widespread phenomenon occurring in more than 80% of

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terrestrial plants (Smith and Read, 2008). AMF enhance nutrient, particularly phosphorus (P), and water uptake by acacia species and improves their nitrogen fixation which enables them to establish them in marginalized lands in the tropics (Requena et al., 2001). These associations contribute to their tolerance to drought, and induce resistance against soil pathogens (Smith and Read, 2008). These associations, in general, enable many of the acacia species perform well in degraded soils with high acidity, high salinity, high aluminum saturation and low soil fertility (Craig et al., 1991). Consequently, acacia trees/shrubs are integrated in the traditional agroforestry systems and for the rehabilitation of fast disappearing and marginalized agro-ecosystems in the tropics and subtropics (Ngulube et al., 1993).

In Ethiopia, the genus *Acacia* is the third dominant group of woody leguminous plants, represented by more than 49 indigenous species, and widely distributed in altitudes ranging from 0 to 3400 m a.s.l (Hunde and Thulin, 1989). The legume is the most important component of the acacia woodland, which is the major vegetation from the arid and semi- arid parts of Ethiopia being utilized in many different ways for the rural economy, and to rehabilitate and stabilize degraded ecosystem especially in the Rift Valley of Ethiopia (Eshete and Stahl, 1999). Although, several studies have been carried out in relation to diversity and density of AMF on coffee and shade trees in montaine forests (Wubet et al., 2003; Muleta et al., 2007), and in the dry deciduous woodlands of Northern Ethiopia (Birhane et al., 2010), studies on the AMF-acacia relationship was limited to the co-inoculation of AMF and rhizobia. The AMF species found in earlier studies of acacia trees belong mainly to the genera *Glomus* and *Gigaspora* (Michelson, 1993; Yohannes and Assefa, 2009). However, currently there is an increase in the land use change for crop production in the country.

The Rift Valley area is one of the regions that suffer most from rapid deforestation that has led to the decrease in the biodiversity of the woodlands for intensive agriculture and settlement for the ever increasing small-hold farming community (Garedew et al., 2009). It is also established that agricultural development can change the whole spectrum of AMF associations that are specifically associated with fitness of specific plants, plant community structure and ecological variability (Van der Heijden et al., 1998). Sanders et al. (1996) reported that plant species respond differently to different AMF species and, density and diversity of naturally occurring AMF were reduced, particularly in disturbed arid and semi arid habitats (Mason and Wilson, 1994). Oehl et al. (2003) also reported that increased land use intensity was correlated with a decrease in AMF species richness and with a preferential selection of species in agro-ecosystems of Central Europe. Another study in Mexican also showed that land use change from temperate forest to avocado plantation had minimal effect on AMF communities, but conversion of forests to maize fields reduced AMF diversity (González-Cortés et al., 2012). Several studies concerning AMF and land use systems have been conducted in tropical ecosystems.

In a study with *Acacia senegal* in the sahelian regions of Senegal, Ndoye et al. (2012) found that the positive effects of this plant species on AMF spore density and diversity as well as on soil microbial functions can be influenced by land-use systems (plantations versus natural populations of *A. senegal*). In tropical dry ecosystems of Mexico, Gavito et al. (2008) found higher AMF morphospecies richness in primary forests than in secondary forests and pastures. In a Brasilian study (da Silva Sousa et al., 2013), the presence of trees (gliricída and maniçoba) increased sporulation, mycorrhizal colonization and the production of infective propagules of AMF in three land use systems. This, therefore, necessitates exhaustive research on the relationship between land use changes and mycorrhizal diversity and density for, any success in rehabilitation for establishment of seedlings depends upon the mycorrhizas in the terrestrial ecosystems (Wubet et al., 2003).

The objective of this investigation was to study the arbuscular mycorrhizal fungal colonization, spore density and diversity of nine acacia species that grow in different land use systems in woodland and savanna woodland vegetation from parts of arid and semi- arid areas of the Rift Valley of Ethiopia.

MATERIALS AND METHODS

Sampling sites

Acacia trees were sampled from three sites, that is, Addis Ababa (2400 m a.s.l.) and Bishoftu and Zeway in the Rift Valley system (1600 to 1960 m a.s.l.). The Addis Ababa site is a high woodland system, while the Rift Valley sites are naturally characterized by woody grassland dominated by different acacia species (Hunde and Thulin, 1989). The sampling areas were represented by six different land use types and vegetation cover. The Addis Ababa site was a (1) protected park (PP). Land use types occurring at Zeway were (2) sorghum cropping in agro-forestry system (SCAFS), (3) protected forest relics managed by Hawassa University (PFR-HU), (4) protected forest relics with natural vegetation (PFRNV) and (5) open grazing field (OGF). The land use type at Bishoftu was a (6) community preserved forest relics for reforestation programmes (CPFR) (Figure 1 and Table 1). Nine acacia tree species (*Acacia abyssinica*, *A. nilotica*, *A. robusta*, *A. saligna*, *A. senegal*, *A. seyal*, *A. sieberiana*, *A. tortilis* and *Faidherbia albida*) were studied. Of these *A. saligna* is an Australian origin while all the other ones are native to Africa. Sampling was conducted during dry seasons from November 2010 to December 2011. The average annual temperature and precipitation of Addis Ababa, Bishoftu and Zeway were 17, 20 and 22°C, and 44, 35 and 74 mm, respectively.

Soil and root sampling

Three separate sample locations were established at each land use type in each site. Within each of these locations (approximately 100

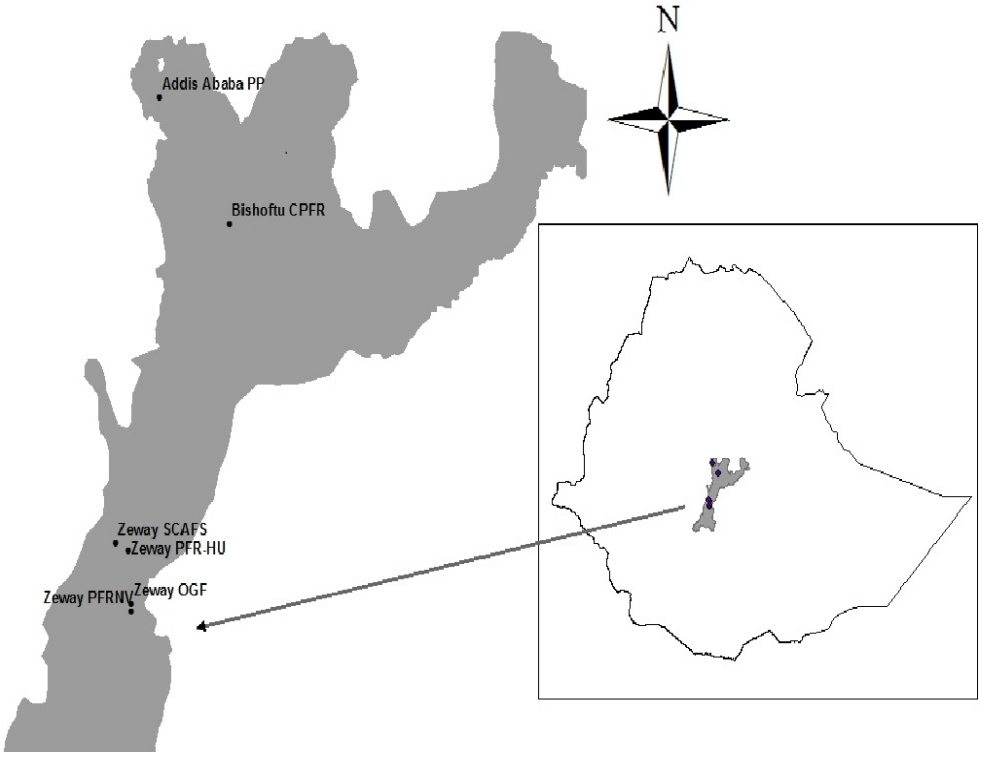

Figure 1. Map of the study site and sampling location.

Table 1. List of acacia species studied from different land use types of the sampling sites.

Name of the acacia species	Plant density/100 m^2	Agro-ecology	Altitude (m)	Sampling sites	Land use type
A. abyssinica	2	Woodland and forest margins	2400	Addis Ababa	PP
F. albida	10	Wooded grassland	1661	Zeway	SCAFS
A. nilotica	15	Wooded grass land	1660	Zeway	PFR-HU
A. senegal	15				
A. tortilis	15	Wooded grass land	1650	Zeway	PFRNV
A. seyal	10				
A. tortilis	10	Wooded grass land	1651	Zeway	OGF
A.seyal	10				
A. sieberiana	12				
A. saligna	15	Wooded grass land	1954	Bishoftu	CPFR
A. seyal	15				
A. robusta	10				

m: Meter; PP*:* protected park; SCAFS: sorghum cropping in agro-forestry system; PFR-HU: protected forest relics managed by Hawassa University; PFRNV: protected forest relics with natural vegetation; OGF: open grazing field; CPFR: community preserved forest relics for reforestation programmes.

m^2), three replicates of each acacia tree species were randomly selected and 500 g of rhizosphere soil and fine roots of the selected trees were taken into a depth of 30 cm and that were pooled into a composite sample per location (Table 1). The samples were collected in alcohol sterilized plastic containers and stored at room temperature until further analysis. Root samples were washed with

tap water and stored in 50% of alcohol at 4°C before determination of root colonization by AM fungi. Soil chemical and physical parameters such as pH, organic carbon (OC), total nitrogen (TN), available phosphorus (P) and soil texture were determined using standard methods at the Addis Ababa city administration environmental protection authority (Table 2). Voucher specimens of the acacia trees were brought and deposited at the National Herbarium, Addis Ababa University for identification.

Assessment of AMF root colonization

The stored root samples were washed carefully with tap water and cut into segments about 1 cm long. About 0.5 g of root segments were cleared in 10% (w/v) KOH at 90°C in a water bath for 2 to 3 h depending on the structure of the root and its pigmentation (Brundrett et al., 1996). Dark roots were further bleached with alkaline hydrogen peroxide (10% H_2O_2) for 3 min at room temperature. Thereafter, the roots were treated with 10% HCl (v/v) for 15 to 20 min at room temperature and finally stained in 0.05% w/v trypan blue in lactoglycerol (1:1:1 lactic acid, glycerol and water) at 90°C for 30 min in a water bath (Brundrett et al., 1996). Fungal colonization was quantified using the magnified intersection method of McGonigle et al. (1990) under a compound-light microscope (OLYMPUS-BX51) at a magnification x200. Accordingly, 150 intersections were observed for each sample. The presence of arbuscular mycorrhizal hyphae, vesicles and arbuscules were recorded.

Spore extraction and identification

Soil samples were air-dried before extraction, counting and identification of AM fungal spores. The AMF spores present were morphologically identified at the Department of Microbial, Cellular and Molecular Biology, Addis Ababa University, Ethiopia and Agrifood Research Finland (MTT), Laukaa, Finland. AMF spores occurring in the soil samples were extracted by the wet sieving and decanting method (Gerdemann and Nicolson, 1963), followed by centrifugation in water and in 50% sucrose solution (Walker et al., 1982). Sieves of size of 500, 250 and 50 µm were used for the wet sieving procedure. Spores, spore clusters and sporocarps obtained from 250 and 50 µm sieves were counted and observed by using a dissecting microscope. Thereafter, spores were mounted on slides in polyvinyl-lactic acid-glycerol (PVLG) (Omar et al., 1979) or in PVLG mixed with Melzer's reagent (1:1 v/v). Spores were examined under a compound microscope and identified to the species level or attributed to a specific morphotype.

Identification and classification were based on a current species descriptions and identification manual (Schenck and Perez, 1990), online references of species description INVAM http://invam.caf.wvu.edu, University of Agriculture in Szczecin, Poland http://www.zor.zut.edu.pl/Glomermycota/, Schüßler and Walker (2010) and the Schüßler AMF phylogeny website http://www.lrz.de/~schuessler/amphylo/

Statistical analysis

Spore density (SD) was expressed as the number of AMF spores per gram of soil. Species richness (SR) was measured as a number of AMF species per sample. Isolation frequency (IF) is (the number of samples in which a given species was isolated / the total number of samples) × 100%. Relative abundance of spores (RA) is (the number of spores in a given species / total number of spores) × 100%. The dominant AMF species were determined according to relative abundance (RA>5%) and isolation frequency (IF >50%) (Li et al., 2007). Analysis of variance (ANOVA) and correlation analysis were carried out with the SPSS software package (version 18.0). Transformed data were subjected to one-way ANOVA to test the differences in AM colonization and spore density among plant species. Multiple mean comparisons were performed using Tukey's HSD post hoc test at the 0.05 level of probability with one-way ANOVA. The relationship between AM colonization and spore density as well as spore density, and species richness and soil parameters were determined by Pearson's correlation analysis.

RESULTS

AMF root colonization

Acacia roots showed mycorrhization with typical structures (arbuscules, hyphae and vesicles) except that arbuscules were not detected in *A. senegal* (data not shown). AMF root colonization varied from 12 to 67.3% (Table 3). The highest colonization (67.3%) was found in *A. seyal* from OGF followed by 44% colonization in *A. nilotica* from PFR-HU, whereas, *A. saligna* from CPFR showed the lowest AM fungi colonization (12%). Arbuscule and vesicle colonization were the highest in the roots of *A. seyal* from OGF, 11.8 and 17.3%, respectively. In contrast, low percentages of arbuscules (0%) and vesicles (1.6%) were recorded from roots of *A. senegal* (PFRNV) and from *A. abyssinica* (PP), respectively. The percentage of AMF root colonization of the same species from different land use types did not show significant difference except, that the percentage of vesicles recorded from *A. seyal* (17.3%) at OGF, was significantly higher than that of the same plant species (8%) from PFRNV (Table 3). The data also shows slight but not significant negative correlation between the root colonization levels and the available P concentration in soil ($r = - 0.40$). However, arbuscular colonization was strongly correlated with vesicular and hyphal colonization ($R^2 = 0.76$ and $r^2 = 0.67$, respectively; $p< 0.05$).

AMF spore density and species diversity

Rhizosphere soils from all acacia species in different land use systems harbored high numbers of AMF spores ranging from 3.7 to 15.0 spores g^{-1} soil with an average of 9.9 spores g^{-1} soil (Figure 2). The highest average spore density of 15.0 spores g^{-1} soil was observed under *A. seyal* (OGF), and the lowest of 3.7 spores g^{-1} of soil under *A. nilotica*. Significant difference ($p<0.05$) in spore density was observed between *A. seyal* (15.0 g^{-1}), *A. abyssinica* (7.5 g^{-1}), *A. robusta (*7.3 g^{-1}) and *A. nilotica* (3.7 g^{-1}). Similarly, spore numbers obtained under *A. senegal* (11.9 spores g^{-1} of soil), *A. tortilis* (12.6 g^{-1}) from PFRNV, *A. sieberiana* (11.5g^{-1}) and *A. seyal* (12.7 g^{-1}) from CPFR were significantly different from spore numbers obtained under *A. nilotica* (3.1 spores g^{-1} of soil). Though, not statistically significant there was an indication of slightly higher spore density in the rhizosphere soil of *A. seyal* from OGF (15.0 spores g^{-1} of

Table 2. Physical and chemical parameters of soil samples from the acacia trees.

Name of the acacia species	pH	T.N (%)	Avail. P (ppm)	O.C (%)	C/N	Clay (%)	Silt (%)	Sand (%)	Soil class	Land use type
A. abyssinica	6.8	0.19	24.54	2.95	16	46	20	34	Clay	PP
F. albida	7.2	0.15	5.82	2.02	14	38	26	36	Clay loam	SCAFS
A. nilotica	6.4	0.37	20.68	4.77	13	48	22	30	Clay	PFR-HU
A. senegal	6.6	0.34	4.72	3.72	11	16	30	54	Sandy loam	
A. tortilis	6.4	0.32	4.44	3.65	11	18	38	44	Loam	PFRNV
A. seyal	6.7	0.32	5.88	3.93	12	14	34	52	Loam	
A. tortilis	6.5	0.33	5.42	4.02	12	18	34	48	Loam	OGF
A. seyal	6.6	0.33	5.32	4.02	12	19	33	48	loam	
A. sieberiana	6.4	0.08	13.06	1.58	20	22	24	54	Sandy clay loam	
A. saligna	6.5	0.12	12.88	1.75	15	44	24	32	Clay	CPFR
A. seyal	6.5	0.15	4.47	1.92	13	24	30	46	Loam	
A. robusta	6.5	0.21	13.86	3.15	15	26	24	50	Sandy clay loam	

T.N: Total nitrogen; Avail. P*:* available phosphorus; O.C: organic carbon; C/N: carbon nitrogen ratio; PP*:* protected park; SCAFS: sorghum cropping in agro forestry system; PFR-HU: protected forest relics managed by Hawassa University; PFRNV: protected forest relics with natural vegetation; OGF: open grazing field; CPFR: community preserved forest relies for reforestation programmes.

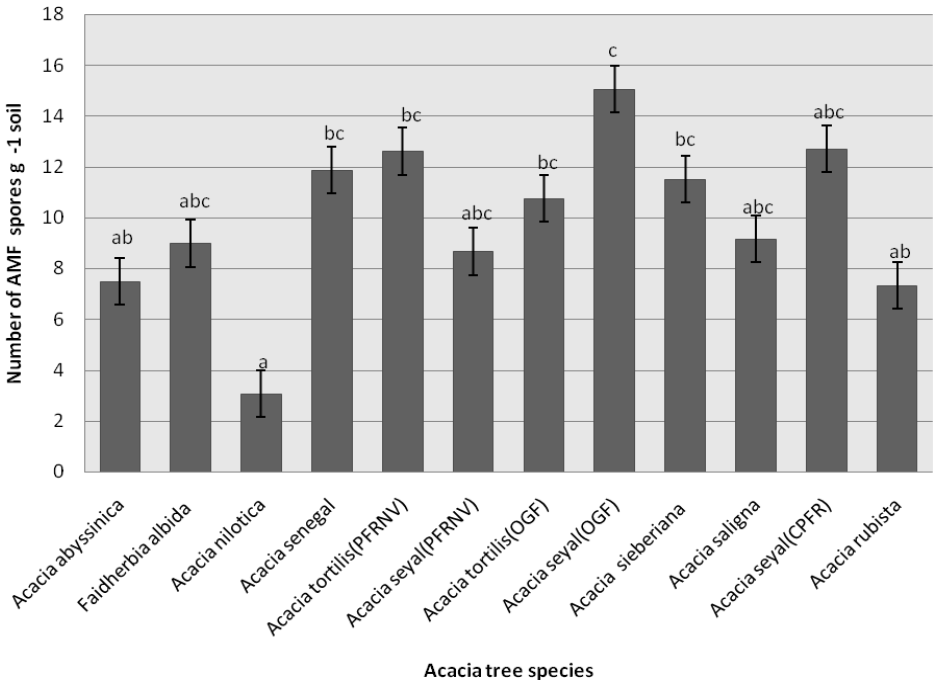

Figure 2. AMF spore abundance in rhizosphere soil of acacia species. Data are reported as (mean ± SE) for three replicate per samples. Significant differences between the samples are indicated by different letters above the bars and were determined by using Tukey HSD at the 0.05 level after one -way ANOVA; PFRNV: protected forest relics with natural vegetation; OGF: open grazing field; CPFR: community preserved forest relies for reforestation programmes.

soil) than in the rhizosphere soil of the same acacia species from forest relics in Bishoftu and Zeway (12.7 spores g^{-1} and 8.7 spores g^{-1} of soil, respectively). Correlation analysis showed a significant negative corre-

Table 3. Percentage of AMF roots colonization of roots in rhizosphere soil of acacia trees.

Name of the acacia species	AM colonization			Land use type
	AC (%)	VC (%)	HC (%)	
A. abyssinica	1.4 ± 0.8[a]	1.6 ± 0.9[a]	15.3 ± 3.8[a]	PP
F. albida	1.7 ±0.3[a]	1.7 ± 0.3[a]	24.5 ± 0.8[a]	SCAFS
A. nilotica	2.8 ± 1[a]	8.9 ± 0.2[ab]	44 ± 1.1[ab]	PFR-HU
A. senegal	0	3.1 ± 1.7[a]	20.2 ± 7.3[a]	
A. tortilis	6.6 ± 1.9[ab]	6.9 ± 1.5[a]	37.6 ± 5.4[ab]	PFRNV
A. seyal	2.8 ± 1[ab]	8 ± 1.1[a]	38 ± 1.7[ab]	
A. tortilis	2.3 ± 0.2[a]	2.3 ± 1.3[a]	37.5 ± 2[ab]	OGF
A. seyal	11.8 ± 3.9[b]	17.3 ± 1.3[b]	67.3 ± 4.4[b]	
A. sieberiana	4.5 ± 2.5[ab]	4.5 ± 2.5[a]	32.3 ± 18[ab]	
A. saligna	2.5 ± 1.4[a]	5 ± 2.8[a]	12 ± 6.9[a]	CPFR
A. seyal	3.3 ± 1.9[ab]	10.3 ± 1.2[ab]	28.5 ± 11[ab]	
A. robusta	2.5 ± 1.4[a]	5.0 ± 2.8[a]	23.7 ± 6.4[a]	

Significant differences between the samples are indicated by different letters in column and were determined by using Tukey HSD at the 0.05 level after one -way ANOVA. AC, VC and HC are percentage of root length with arbuscule, vesicle and hyphal colonization, respectively; PP: protected park; SCAFS: sorghum cropping in agro forestry system; PFR-HU: protected forest relics managed by Hawassa University; PFRNV: protected forest relics with natural vegetation; OGF: open grazing field; CPFR: community preserved forest relies for reforestation programmes.

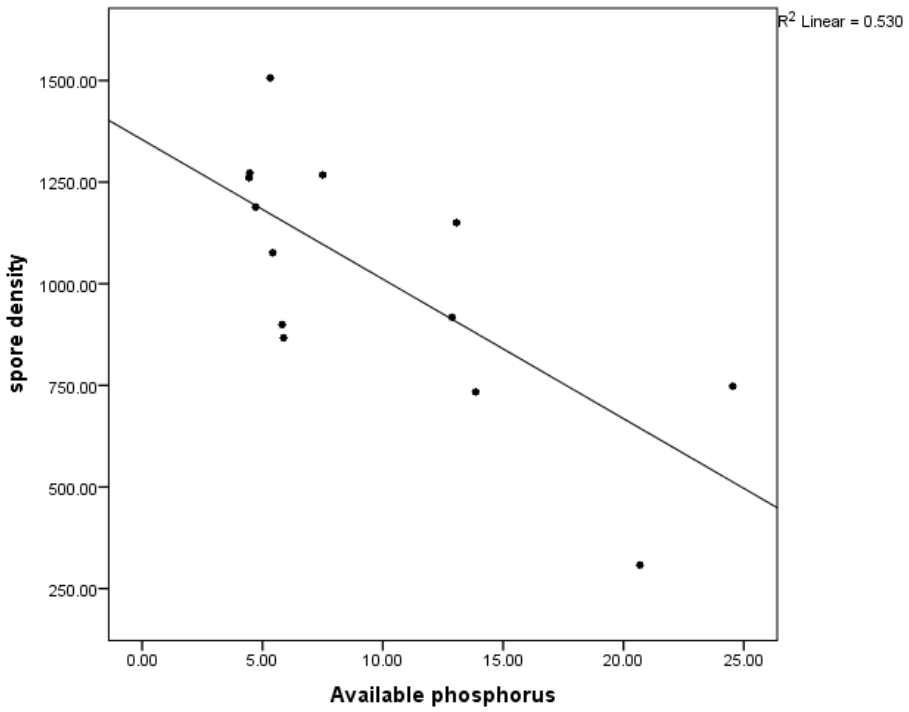

Figure 3. Correlation between available phosphorus and spore density.

lation between AMF spore density and available P in soil (r = 0.-728, p < 0.01) (Figure 3). AMF spore density was positively correlated with the percentage of soil texture such as silt and sand (r = 0.649 and 0.604, p<0.05, respectively), but negatively correlated with the percentage of clay (r = -0.710, p<0.01).

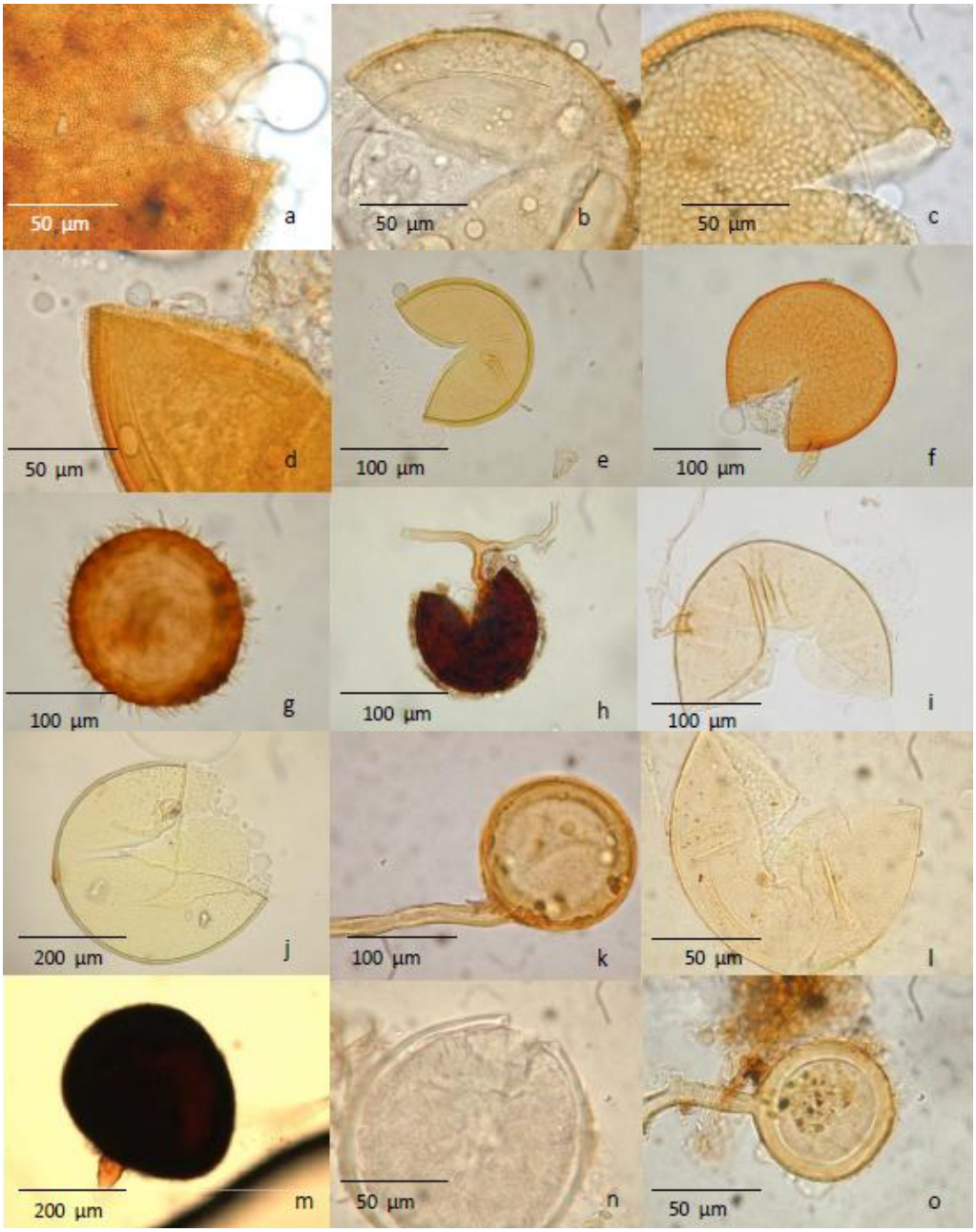

Figure 4. Some Glomeromycotan species identified from rhizosphere soil samples of acacia species in Ethiopia. All photos are from slides made in PVLG. a) *Acaulospora dentidulata*, b) *A. kentiniensis*, c) *A. scrobiculata*, d) *A. spinosa*, e) *Claroideoglomus claroideum*, f) *Diversispora epigaea*, g) *Entrophospora nevadensis*, h) *Funneliformis geosporus*, i) *F. mosseae*, j) *Gigaspora gigantea*, k) *Glomus hoi*, l) *Pacispora scintillans*, m) *Racocetra gregaria*, n) *Rhizophagus diaphanus*, o) *R. fasciculatus.*

A total of 41 AMF species belonging to 14 genera and seven families were identified from all rhizosphere soil samples of acacia species (Table 4 and Figure 4). Nine species belonged to *Acaulospora*, 6 to *Funneliformis*, 4 each to *Gigaspora*, *Glomus*, and *Rhizophagus*, 3 each to *Claroideoglomus*, and *Scutellospora*, 2 each to *Racocetra* and *Diversispora*, and 1 each to *Entrophosphora*, *Sclerocystis*, *Paraglomus* and *Pacispora* (Table 4). Additionally, 2 unidentified morphotypes each of *Glomus*, and *Acaulospora* and 1 of *Archaeospora* were also observed. The highest species diversity of 19 species from 7 genera was detected in the rhizosphere soil of *A. saligna* followed by 18 species from *A. abyssinica* representing 6 genera. The lowest species

Table 4. Identified arbuscular mycorrhizal fungi, their frequency of occurrence and relative abundances in rhizosphere soil of acacia species.

AMF species	*A. abyssinca*	*F. albida*	*A. nilotica*	*A. senegal*	*A. tortilis* (PFRNV)	*A. seyal* (PFRNV)	*A. tortilis* (OGF)	*A. seyal* (OGF)	*A. sieberiana*	*A. saligna*	*A. seyal* (CPFR)	*A. robusta*	IF (%)	RA (%)
Acaulospora													158	6.85
A. capsicula Błaszk.				x									8.3	0.25
A. cavarnata Błaszk.										x			8.3	0.25
A. denticulata Sieverd. & S. Toro		x								x	x	x	33.3	1.79
A. faveata Trappe & Janos	x												8.3	0.25
A. laevis Gerd. & Trappe										x			8.3	0.25
A. rehmii Sieverd. & S. Toro	x		x		x						x		33.3	1.53
A. sorbiculata Trappe							x					x	16.6	1.02
A. spinosa Walker & Trappe	x								x				16.6	0.76
A. tuberculata Janos & Trappe					x								8.3	0.25
Acaulospora sp1										x			8.3	0.25
Acaulospora sp2									x				8.3	0.25
Archaeospora													8.3	0.25
Archaeospora sp								x					8.3	0.25
Claroideoglomus													258	27.1
C. claroideum (Schenck & Sm.) Walker & Schuessler	x	x	x	x		x	x	x	x	x	x	x	91.6	7.16
*C. etunicatum (*Becker & Gerd.) Walker & Schuessler	x	x	x	x	x		x	x		x	x		75	5.37
C. luteum (Kenn, Stutz & Morton) Walker & Schuessler	x	x	x	x	x	x	x	x	x		x	x	91.6	14.57
Diversispora													24.9	1.27
D. celata Walker, Gamper & Schuessler	x												8.3	0.51
D. epigaea (Daniels & Trappe) Walker & Schuessler					x			x					16.6	0.76
Entrophosphora													8.3	0.25
E. nevadensis Błaszk., Madej & Tadych; Palenzuela, Ferrol, Azcón-Aguilar & Oehl							x						8.3	0.25
Funneliformis													316	19.9
F. badius (Oehl, Redecker & Sieverd.) Walker & Schuessler						x		x					16.6	1.53
F. caledonius (Nicolson & Gerd.) Walker & Schuessler	x			x			x		x	x	x		50	1.53
F. constrictus (Trappe) Walker & Schuessler	x	x	x	x					x	x		x	58.3	4.34
F. coronatus (Giovann.) Walker & Schuessler		x										x	16.6	0.76
F. geosporus Nicolson & Gerd.) Walker & Schuessler	x		x	x	x	x	x	x	x	x	x	x	91.6	6.9
F. mossae (Oehl, Redecker & Sieverd.) Walker & Schuessler	x	x	x	x		x	x	x	x	x			83.3	4.85
Glomus													275	24.5

Table 4. Contd.

G. aggregatum Schenck & Sm.	x	x	x	x	x	x	x	x		x	x	x	91.6	13
G. hoi Berch & Trappe										x			8.3	0.25
G. microaggregatum (Koske, Gemma & Olexia)									x	x	x		25	1.02
G. microcarpum Tul. & Tul.	x		x	x	x	x		x					50	4.6
Glomus sp1 (sporocarpic, thick wall & smooth, 80-110 µm)							x		x	x	x	x	41.6	2.81
Glomus sp2 (red brown geosporum like)	x	x		x	x	x		x	x				58.3	2.81
Rhizophagus													66.5	2.54
R. clarus (Nicolson & Schenck) Walker & Schuessler									x				8.3	0.25
R. diaphanus (Morton & Walker) Walker & Schuessler			x							x			16.6	0.76
R. fasciculatus (Thaxt.) Walker & Schuessler					x						x	x	25	1.02
R. intraradices (Schenck & Sm.) Walker & Schuessler	x							x					16.6	0.51
Sclerocystis													25	0.76
S. sinuosa Gerd. & Bakshi	x							x	x				*25*	*0.76*
Gigaspora													58.2	2.28
G. albida Schenck & Sm				x			x	x					25	1.02
G. decipiens Hall & Abbott							x						8.3	0.25
G. gigantea (Nicolson & Gerd.) Gerd. & Trappe						x				x			16.6	0.76
G. margarita Becker & Hall		x											8.3	0.25
Racocetra													41.6	2.55
R. fulgida (Koske & Walker) Oehl, Souza & Sieverd.		x											8.3	0.25
R. gregaria (Schenck & Nicolson) Oehl, Souza & Sieverd.		x		x			x	x					33.3	2.3
Scutellospora													108	6.59
S. calospora Nicolson & Gerd.) Walker & Sanders				x			x			x			25	1.79
S. cerradensis Spain & Miranda			x	x	x			x	x				41.6	2.3
S. pellucida (Nicolson & Schenck) Walker & Sanders		x		x		x	x		x				41.6	2.5
Pacispora													41.6	3.06
P. scintillans (Rose & Trappe) Walker, Vestberg & Schuessler	x		x					x		x	x		41.6	3.06
Paraglomus													41.6	1.79
P. occultum (Walker) Morton & Redecker	x	x				x	x			x			41.6	1.79

PFRNV: Protected forest relies with natural vegetation; OGF: open grazing field; CPFR: community preserved forest relies.

diversity of 10 was detected from *A. robusta*, in 5 genera, and 11 species each from *A. tortilis* and *A. seyal*, with 6 and 5 genera, respectively (Table 5). Among land use types, the highest numbers of 31 AMF species in 9 genera were detected in CPFR at Bishoftu and the lowest numbers of 6 species in 6 genera were in PFR-HU at Zeway (Table 5). Based on relative abundance and isolation frequency of spores, the 5 dominant species identified were *C. claroideum*, *C. etunicatum*, *C. luteum*, *F. geosporus* and *G. aggregatum* (Table 4).

Generally, 32.6, 19.5 and 19.5% of AMF species were identified from the families of *Glomeraceae*, *Acaulosporaceae* and *Gigasporaceae*, respectively. However, most AMF species from the

Table 5. Summary of total AMF genera and species identified in the plant species and land use types.

Plant species	Total AMF identified from the plants		Land use type	Total AMF identified in the land use type	
	Genera	Species		Genera	Species
A. abyssinica	8	18	PP	8	8
F. albida	8	14	SCAFS	8	8
A. nilotica	6	12	PFR-HU	6	6
A. senegal	7	16			
A. tortilis	6	11	PFRNV	9	22
A. seyal	5	11			
A. tortilis	9	16	OGF	11	25
A. seyal	9	17			
A. sieberiana	7	15			
A.saligna	7	19	CPFR	9	31
A.seyal	6	12			
A. robusta	5	10			

PP: Protected park; SCAFS: sorghum cropping in agro-forestry system; PFR-HU: protected forest relics managed by Hawassa University; PFRNV: protected forest relics with natural vegetation; OGF: open grazing field; CPFR: community preserved forest relies for reforestation programmes.

Acaulosporacae had low relative abundance and frequency of isolation compared to the other families.

DISCUSSION

In this study, we report AMF spore density, diversity and root colonization of selected species of acacia growing in different land use types in Ethiopia (Table 1). AMF were present in all roots and rhizosphere soil samples of acacia trees with low (12%) to moderate (67.3%) levels of colonization. This pattern is similar to that observed in other tropical systems such as (0 to 75% colonization) in acacia and other woody legume species in dry deciduous forest areas of Northern Ethiopia (Birhane et al., 2010), (31 to 64% colonization) in acacia and prosopis tree species in Senegal (Ingleby et al., 1997), (35 to 65% colonization) in acacia tree species, in India (Lakshman et al., 2001) and (56 to 73% colonization) in *A. farnesiana* and *A. planifrons*, in India (Udaiyan et al., 1996). This study showed intra- and interspecific variations in AM colonization among acacia plants (Table 3).

The AM colonization reported in acacia species in our study supports the view that legumes have a high mycorrhizal dependency, as pointed out by Plenchette et al. (1983). Correlation analysis showed that arbuscular colonization was positively correlated with hyphal and vesicular colonization (Lingfei et al., 2005). Concerning soil parameters, though not significant at $p<0.05$, there was an indication of positive correlation between percentages of hyphal colonization and organic carbon (0.536) and a negative correlation between hyphal colonization and available P (-0.454), a result that is similar to the work of Lingfei et al. (2005). Also, Kahiluoto et al. (2001) suggested a negative correlation between available phosphorus and AM colonization.Significant variation in the abundance of AMF spores was found in the rhizosphere soil of acacia tree species in the same or different plant community (Figure 2). The mean number of spores per 100 g of soil ranged from 307 to 1506 with an average of 994. Other studies in similar or different host plants of the tropical area corroborate our finding: 775 to 1240 spores 100 g^{-1} soil in *A. albida* Del. in Senegal (Diop et al., 1994); 500 to 1500 spores 100 g^{-1} soil in *A. farnesiana* and *A. planifrons* in moderately fertile alkaline soils in India (Udaiyan et al., 1996); 110 to 2600 spores 100 g^{-1} soil in tropical forest and pasture (Picone, 2000) and 5 to 6400 spores 100 g^{-1} soil in a valley savanna of the dry tropics (Tao et al., 2004). By contrast, low spore densities of 11 to 32 spores 100 g^{-1} soil were detected in dry deciduous woodlands of Northern Ethiopia associated with different acacia species (Birhane et al., 2010).

Low AMF spore numbers were also recorded in a survey of acacia tree species (49 to 67 spores 100 g^{-1} soil) in India (Lakshman et al., 2001) and in acacia and prosopis tree species (8 to 51 spores 100 g^{-1} soil) in Senegal (Ingleby et al., 1997). The variation in AMF spore density between samples could be due to factors such as climatic and edaphic properties, spatial and temporal variation, vegetation, host-specificity between fungi and plants, age of the host plants, disturbance, and differential sporulation ability of AMF taxa (Husband et al., 2002; Muthukumar and Udaiyan, 2002).

The highest numbers of AMF spores were recorded in the rhizosphere soil of *A. seyal* from the open grazing

field at Zeway, and these numbers were also higher than the same species at Zeway and at Bishoftu protected forest relics. In addition, the spore count obtained in the rhizosphere soil of *A. seyal* from OGF was highly significantly greater than the spore count in *A. nilotica* from PFR-HU, *A. abyssinica* from PP and *A. robusta* from CPFR, respectively (Figure 2). According to Janos (1992) and Picone (2000), disturbed ecosystems induce AMF to sporulate because of grazing, disturbance and slow rate of decomposition. This is however in contrast to the conclusion of Birhane et al. (2010), who suggested that management in the form of exclosure (that excludes grazing) had a positive effect on spore abundance. The percentage of root colonization and the number of AMF spores observed in the sampled acacia trees did not correlate significantly ($r = 0.48$, $p>0.05$). This finding is in line with results obtained by Becerra et al. (2009). The relationship between AMF spore density and percentage of root colonization are influenced by many biotic and abiotic environmental factors such as AM fungal species, plant host and soil nutrients (Stutz and Morton, 1996). The present study showed a significant negative correlation between spore density and available P ($r = -0.728$, $p< 0.01$, Figure 3), which is similar to some reports from India and Northern Europe (Udaiyan et al., 1996; Kahiluoto et al., 2001). The decrease in spore density with an increase in soil available P observed in the study can be attributed to the fact that, available soil phosphorus suppresses AM root colonization as well as AMF density (Kahiluoto et al., 2001).

In contrast to our finding, Muleta et al. (2007) observed a positive relationship between spore number and available P in soil samples from natural coffee forest inEthiopia. They suggested that the characteristics of available P level in their study were not high enough to influence mycorrhizal development. As far as soil texture is concerned, spore density showed a significant positive correlation with sandy soil and negative correlation with clay soil ($r = 0.604$ and -0.710; $p< 0.05$ and 0.01, respectively). This result is in line with the findings of Carrenho et al. (2007) who suggested that sandy soil stimulated the development of mycorrhizal association while clay soil inhibited it. This may be because sandy soils are usually more porous, warmer, drier, and less fertile than those of a finer texture and these conditions have direct and indirect effects on AMF (Sylvia and Williams, 1992). In this study, both high spore density and diversity AMF species were observed in the rhizosphere of acacia trees. Based on spore morphology, 41 AMF species and 5 morphotypes were identified (Table 4). Similarly, 44 and 60 AM fungal species were detected from semiarid grasslands of Namibia (Uhlmann et al., 2004) and sub-Saharan Savannas of Benin, West Africa (Tchabi et al., 2008), respectively. Likewise, 43 species of AMF were isolated from Western Brazilian Amazon (Stürmer and Siqueira, 2011).

The high AMF species richness observed in our acacia study is in contrast to the only 17 AMF species isolated from tropical humid high land of Kenya (Jefwa et al., 2009). Mathimaran et al. (2007) found 18 species in Kenyan ferralsol soil and Emmanuel et al. (2010) recorded 17 AMF species from soil fertility management systems in Nigeria. According to Helgason et al. (1998), woodlands have higher AM fungal species richness and diversity compared to agricultural ecosystems. Generally, *Acaulospora* and *Funneliformis* were the dominant genera accounting for 9 and 6 species, respectively. This result is similar to that observed in tropical systems in the hot-dry valley of the Jinsha River, Southwest China (Zhao and Zhao, 2007), in Panama (Mangan et al., 2004), and in Brazil (Stürmer et al., 2006). The dominance of these two genera may be related to their sporogenous characteristics, that is, the production of relatively small spores within a short period of time compared with the large spores of *Gigaspora* and *Scutellospora* genera (Hepper, 1984; Bever et al., 1996). Among the 41 species identified, the most frequently encountered were *Claroideoglomus luteum*, *Glomus aggregatum*, *C. claroideum*, *C. etunicatum* and *Funneliformis geosporus* (Table 4). Other studies have also shown that these species are repetitively detected in temperate and tropical ecosystems (Stutz et al., 2000; Oehl et al., 2003; Zhao and Zhao, 2007). On the other hand, AMF species such as *G. hoi*, *A. capsicula*, *A. cavarnata*, *A. faveata*, *A. tuberculata*, *Diversispora celata*, *Entrophospora nevadensis*, *Rhizophagus clarus*, *G. decipiens*, *G. margarita* and *Racocetra fulgida* occurred only in one of the acacia species suggesting that they are specific in their distribution.

We found a significant positive correlation between relative abundance and isolation frequency of AMF species ($r = 0.881$, $p< 0.01$) indicating that species producing more spores usually had a wider distribution, while species with small geographic ranges usually produced fewer spores (Zhao and Zhao, 2007). Within the limits of the sampling areas, our study showed relatively high mycorrhizal colonization but very high AMF species diversity in the rhizosphere soil of different acacia species growing in Ethiopia. The study also indicated that AMF spore density and the extent of AMF colonization in acacia roots was influenced by soil factors such as available P and soil texture. The results obtained have wider implications for improving restoration success of soil fertility in degraded soils. Our small-scale field survey confirms that attention should be given to woody legumes of high mycorrhizal dependency that have high agroforestry importance in the country.

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Full Length Research Paper

Phylogeny analysis of Indian strains of *Rhizoctonia solani* isolated from chickpea and development of sequence characterized amplified region (SCAR) marker for detection of the pathogen

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Wet root rot caused by *Rhizoctonia solani* is one of the important diseases in chickpea worldwide. In the present study, 10 random amplified polymorphic DNA (RAPD) primers were used to assess the molecular diversity of 50 chickpea isolates of *R. solani.* There was a great diversity among the isolates studied and was in the range of 52 to 93%. The isolates were highly variable in aggressiveness and caused up to 100% wet root rot incidence in chickpea. Accurate detection and identification of plant pathogens are fundamental to plant pathogen diagnostics and management. Therefore, a polymerase chain reaction (PCR) assay was developed for accurate and sensitive detection of *R. solani* from mycelial DNA and infected chickpea plants. RAPD primer OPA 11 consistently amplified ≈1700 base pairs (bp) product in PCR only from the DNA of *R. solani* isolated from chickpea. The common DNA fragment was sequenced and used to design a pair of oligonucleotide primers amplifying 285 bp sequence characterized amplified region (SCAR). The specificity of the SCAR primers was evaluated. The detection sensitivity of *R. solani* was 0.5 ng for the genomic DNA and 5 ng for the DNA extracted from infected chickpea root samples. Also, SCAR primer was validated with Q-PCR to detect and quantify *R. solani* upto 1 pg from infected chickpea root samples. These new SCAR marker are useful for early detection and quantification of wet root rot pathogen in chickpea.

Key words: Anastomosis grouping, chickpea, wet root rot, quantitative-PCR (Q-PCR), random amplified polymorphic DNA (RAPD), sequences characterized amplified region (SCAR).

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a leading winter legume crop in India. It is cultivated in an about 8.21 million hectares, with an average annual production of 7.48 million tonnes along with a productivity of 911 kg ha^{-1} (Anonymous, 2010). Besides in India, chickpea is widely cultivated in other tropical, sub-tropical and temperate regions of the world. *Kabuli* type chickpea is grown in temperate regions while the *desi* type is grown in semi arid tropics. Both types of chickpea are commonly grown in India, but the cultivation of *desi* type is predominated. All types of chickpea irrespective of plant type, variety and seed size are susceptible to *Rhizoctonia solani*. Many soil borne fungal pathogens cause wilts and root rots in chickpea, which seriously reduce the production. Among all, wet root rot (WRR) caused by *R. solani* Kühn, is important production constraints in chickpea cultivation especially in rice-chickpea cropping system or in wet areas (Haware, 1998). *R. solani* is a polyphagous fungus,

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very common in most soils and with a very wide range of host plants (Nelson et al., 1996; Blazier and Conway, 2004; De Curtis et al., 2010). *R. solani* causes root rot in many pulse crops when they are weakened by other stress factors (Singh and Mehrotra, 1982). WRR is an important disease affecting seedling establishment of chickpea which leads to the reduction of chickpea yields in Canada (Hwang et al*.,* 2003). This disease is most commonly observed at early in the season when soil moisture content is often high; however, it also can be observed any time during the season.

All the *R. solani* strains vary in cultural appearance, anastomosis groupings (AGs), virulence and physiology (Parmeter and Whitney, 1970). Many scientists tried to organize *R. solani* isolates into groups on the basis of various morphological, physiological, pathological characteristics (Sherwood, 1969) and anastomosis behaviour (Parmeter et al*.,* 1969; Ogoshi, 1987). The major drawbacks in morphological characteristics of the pathogen are the reliance on the ability of the organism to be cultured, the time consumption, labor intensive nature, and the requirement for skilled taxonomical expertise (Lievens et al*.,* 2005). Grouping of *R. solani* based on AG's, is time consuming because the amount of time for anastomosis to occur is not predictable and hyphal overlapping (Zhang and Dernoeden, 1995).

To avoid the above limitations, a new era of DNA based markers system (De Curtis et al., 2004; Schena et al., 2004) begun with the RAPD markers (Williams et al., 1990). Random amplified polymorphic DNA (RAPD) offers a promising, versatile and informative molecular tool to detect genetic variation within population of plant pathogens (Sharma et al., 2005).

The identification of WRR infected chickpea plant is very complicated because many soil borne fungal pathogens such as *Fusarium* species*, Sclerotinia sclerotiorum, Rhizoctonia bataticola, Pythium* spp. show similar symptoms like gradual yellowing, wilting of foliage and root rotting. Early and accurate detection and identification of plant pathogens are essential for effective plant disease management. Molecular techniques can overcome many of the shortcomings of the conventional assays, espe-cially if they make use of the PCR (Lievens et al*.,* 2005).

RAPD is frequently used for genetic diversity analysis of fungal pathogens, owing to its simplicity, low cost and lower infrastructure requirements (Sharma et al*.,* 2005). However, the fragment polymorphisms used in RAPD markers are not always reproducible (Shimada et al., 2008). This limitation can be overcome by converting RAPD's into sequence-characterized amplified region (SCAR) markers. Compared with conventional PCR for pathogen detection, RT-PCR is highly sensitive, quick and efficient molecular technique for the gel free detection of many plant pathogenic fungi from infected plant and soil samples (Schena et al., 2002; Schena and Ippolito, 2003; Sayler and Yang, 2007; Brierley et al., 2009; Shishido et al., 2010; Guo et al., 2012). A molecular marker for detection of *R. solani* in chickpea is lacking. The present study was aimed to characterize the chickpea population of *R. solani* and to develop a reproducible and sensitive SCAR marker for detection of *R. solani* from infected chickpea plants and this primer was further validated with Q-PCR.

MATERIALS AND METHODS

Fungal cultures

Fifty (50) isolates of *R. solani* representing major chickpea growing areas of India were collected from the Pulse laboratory, Division of Plant Pathology, IARI, New Delhi, India (Table 1) for the present study. The isolates were purified by single hyphal tip culture on 1.5% water agar and were transferred to potato dextrose agar (PDA) medium (Himedia, India). Pure cultures of different isolates of *R. solani* were maintained at 25±1°C on PDA slants for further studies. The cultures of *R. bataticola*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum* f sp *ciceris* and *Pythium aphanidermatum* were also obtained from the Pulse laboratory, Division of Plant Pathology, IARI, and New Delhi, India and maintained on PDA medium for the study.

Aggressiveness of the isolates

Pot experiment in net house was conducted to determine the aggressiveness of the isolates of *R. solani* included in the present study. Surface sterilized (0.1% formalin) plastic pots (20 cm) were filled (2 kg pot^{-}1) with sterilized soil (1% formalin). The soil was inoculated 2-days prior sowing with 10-day-old inoculum (10 g kg^{-}1 soil) of *R. solani* multiplied on sorghum grains (Dubey et al., 2009). Ten seeds of chickpea variety JG 62 were sown in each pot during winter season of 2010-2011 in three replications. The incidence of wet root rot was recorded at 15 days interval up to maturity of the crop plants. The data were analyzed statistically in completely randomized design (Gomez and Gomez, 1984) using Windostat version 7.0 (Indostat Services, Hyderabad, India). The statistical significance was assessed at $p<0.05$ and Fisher's least significant difference test was used to separate means.

Extraction of DNA from *R. solani*

For DNA extraction, mycelial cultures of the isolates of *R. solani* and chickpea pathogenic fungi used in the present study were grown in PDA (Himedia, India) for five days in incubator shaker (120 rpm at 25±1°C). Mycelium was harvested and DNA was extracted according to standard protocols (Murray and Thompson, 1980). The mycelium (1 g) was collected with a pre-cooled mortar and pestle and mixed with pre-warmed (65°C) 2% CTAB DNA extraction buffer. The tubes were incubated in a water bath at 65°C for 1 h with gentle shaking at every 10 min intervals. After incubation and cooling at room temperature, an equal volume of phenol/-chloroform/isoamyl alcohol (25:24:1) was added and mixed gently to denature proteins and centrifuged at 12,000 rpm at room temperature for 20 min. The aqueous phase was transferred to a new sterile tube and equal volume of chloroform/isoamyl alcohol (24:1 v/v) was added and mixed gently and centrifuged at 10,000 rpm for 10 min. The aqueous phase was transferred to a new sterile tube and last step was repeated once again to get pure DNA. The aqueous phase was transferred to a new tube and DNA was precipitated with 0.6 volume of ice cold isopropanol and 0.1 volume

Table 1. The isolates of *Rhizoctonia solani* used in the present study indicating their accession numbers, place of collection, anastomosis groups and disease incidence caused by them.

Accession number	Plant parts	District	State	AG Group	Disease incidence (%)	Aggressive group
RTNG 4	Root	Coimbatore	Tamil Nadu	AG 5	81.8 (64.72) [c]	LA
RTNG 5	Root	Thiruppur	Tamil Nadu	Unknown	54.1 (47.59) [g]	HA
RTNG 6	Root	Coimbatore	Tamil Nadu	Unknown	45.1 (42.37) [h]	MA
RTNG 7	Root	Dharmapuri	Tamil Nadu	AG 1	62.5 (52.49) [f]	HA
RTNG 8	Root	Dharmapuri	Tamil Nadu	AG 1	54.5 (47.78) [g]	HA
RKNG 9	Root	Dharwad	Karnataka	AG 1	44.6 (42.94) [h]	MA
RKNG 10	Root	Bangaluru	Karnataka	AG 2-2 LP	17.4 (25.48) [m]	LA
RKNG 11	Root	Bangaluru	Karnataka	AG 4	64.5 (53.08) [e]	HA
RAPG 9	Root	Kurnool	Andhra Pradesh	AG 2-2	35.5 (37.07) [i]	MA
RAPG 11	Root	Kurnool	Andhra Pradesh	AG 3	26.0 (31.47) [k]	MA
RAPG 13	Root	Kurnool	Andhra Pradesh	AG 2-3	90.1 (72.41) [b]	LA
RAPG 14	Root	Kurnool	Andhra Pradesh	AG 2-3	9.1 (17.55) [n]	LA
RAPG 15	Root	Hyderabad	Andhra Pradesh	AG 4	35.8 (37.07) [i]	MA
RAPG 16	Root	Hyderabad	Andhra Pradesh	AG 2-2 LP	43.5 (42.75) [h]	MA
RMHG 23	Root	Pune	Maharashtra	AG 3	100.0 (80.94) [a]	HA
RMHG 24	Root	Pune	Maharashtra	AG 1	25.8 (31.25) [kl]	MA
RMHG 25	Root	Pune	Maharashtra	AG 4	26.8 (31.47) [kl]	MA
RMHG 28	Root	Ahmadnagar	Maharashtra	AG 5	43.5 (41.79) [h]	MA
RMHG 31	Root	Jalgaon	Maharashtra	AG 1	17.7 (25.24) [m]	LA
RMHG 32	Root	Jalgaon	Maharashtra	AG 3	44.4 (42.94) [h]	MA
RMHG 33	Root	Jalgaon	Maharashtra	AG 3	25.5 (31.89) [kl]	MA
RMHG 35	Root	Jalgaon	Maharashtra	Unknown	9.0 (17.55) [n]	LA
RRJG 1	Root	Sriganganagar	Rajasthan	AG 5	62.2 (52.49) [f]	HA
RRJG 3	Root	Sriganganagar	Rajasthan	Unknown	71.5 (58.49) [d]	HA
RRJG 4	Root	Hanumangarh	Rajasthan	AG 2-3	72.8 (58.06) [d]	HA
RGJG 1	Root	Ahmedabad	Gujarat	AG 3	90.3 (73.45) [b]	HA
RGJG 2	Root	Ahmedabad	Gujarat	AG 5	89.9 (72.41) [b]	HA
RGJG 4	Root	Ahmedabad	Gujarat	Unknown	35.5 (37.47) [i]	MA
RGJG 5	Root	Kheda	Gujarat	AG 3	24.3 (31.47) [kl]	MA
RGJG 6	Root	Dahod	Gujarat	AG 5	100.0 (83.9) [a]	HA
RGJG 7	Root	Dahod	Gujarat	AG 5	54.5 (47.59) [g]	HA
RUPG 96	Root	Mirzapur	Uttar Pradesh	Unknown	99.3 (90.00) [a]	HA
RUPG 97	Root	Mirzapur	Uttar Pradesh	AG 4	35.8 (37.07) [i]	MA
RUPG 98	Root	Sonebhadra	Uttar Pradesh	AG 3	71.5 (58.49) [d]	HA
RUPG 99	Root	Sonebhadra	Uttar Pradesh	AG 2-3	35.8 (37.27) [i]	MA
RUPG 100	Root	Sonebhadra	Uttar Pradesh	AG 3	26.8 (31.47) [kl]	MA
RUPG 103	Root	Jhansi	Uttar Pradesh	AG 5	8.6 (17.87) [n]	LA
RUPG 106	Root	Jhansi	Uttar Pradesh	AG 3	8.0 (18.18) [n]	LA
RUPG 107	Root	Jhansi	Uttar Pradesh	AG 2-2	8.6 (18.14) [n]	LA
RMPG 28	Root	Damoh	Madhya Pradesh	AG 2-3	28.6 (32.74) [j]	MA
RMPG 31	Root	Chattarpur	Madhya Pradesh	AG 5	35.5 (37.07) [i]	MA
RHRG 5	Root	Mahendragarh	Haryana	AG 2-3	81.8 (64.47) [c]	HA
RHRG 7	Root	Mahendragarh	Haryana	AG 3	36.1 (37.07) [i]	MA
RHRG 8	Root	Bhiwani	Haryana	AG 5	25.5 (31.68) [kl]	MA
RHRG 9	Root	Bhiwani	Haryana	AG 2-2 LP	90.1 (71.77) [b]	HA
RHRG 11	Root	Bhiwani	Haryana	AG 3	9.4 (17.87) [n]	LA
RHRG 13	Root	Bhiwani	Haryana	AG 5	55.5 (47.78) [g]	HA

Table 1. Contd

RHRG 14	Root	Bhiwani	Haryana	AG 3	99.3 (85.68) [a]	HA
RHRG 15	Root	Bhiwani	Haryana	AG 3	55.2 (47.59) [g]	HA
RDLG 3	Root	New Delhi	Delhi	AG 3	55.2 (47.97) [g]	HA

LA, less aggressive (mortality ≤ 20%); MA,moderately aggressive (mortality > 20 to 50%); H, highly aggressive (mortality > 50%). Figures in parentheses are transformed angular values. The values within a column with different letters are significantly different at 5% level by using Fisher's least significance difference test.

Table 2. Primer sequence, number of polymorphic bands, percentage of polymorphism and range of amplicons size obtained from RAPD markers.

Primer	Sequence (5'-3')	Annealing temperature (°C)	Total number of bands	% polymorphism	Range of amplicons size (kb)
A03	CGACGACGACGA	35	7	100	0.25- 2.0
A08	GCCCCGTTAGCA	35	7	100	0.5- 3.0
OPA3	AGTCAGCCAC	35	9	100	0.3- 3.0
OPA11	CAATCGCCGT	35	8	87.5	0.3- 4.0
OPA18	GACCGCTTGT	35	10	100	0.5- 3.0
OPD4	TCTGGTGAGG	35	8	100	0.5- 3.0
OPN20	GACCGACCCA	35	9	100	0.3- 3.0
P14	CCACAGCACG	35	8	100	0.5- 2.5
R1	GTCCATTCAGTCGGTGCT	35	9	100	0.25- 3.0
R28	ATGGATCCGC	35	10	100	0.5- 2.5

of 3 M sodium acetate and allowed to precipitate at -20°C for 3-4 h, followed by centrifuging at 10,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was washed twice with 70% ethanol and dried at either room temperature or 37°C. The DNA pellet was resuspended in 100 to 200 µL TE buffer and stored at -20°C for further use.

RAPD analysis

Ten (10) RAPD primers were obtained from Sigma, Bangalore used in PCR (Table 2). Amplification reactions were done in a 25 µL reaction mixture containing 50 ng template DNA, 1.5 U Taq DNA polymerase (Bangalore Genei, India), 3.5 mM of $MgCl_2$, 0.6 mM of each dNTPs (Bangalore Genei, India), and 10 pmol of primer in 1X reaction buffer. PCR was performed by using gradient thermal cycler (Eppendorf ep^{TM}, Germany) with 94°C for 5 min for initial denaturation followed by 40 cycles at 94°C for 1 min denaturation, annealing at 35°C for 1 min, and extension at 72°C for 2 min with a final elongation of 72°C for 5 min. Amplified products were analyzed by electrophoresis in 1.0% agarose gel in 1X TAE buffer. A 1 kb ladder (Bangalore Genei, India) was used as a marker. Gels were stained with ethidium bromide (1 µg/mL) and observed under UV light in gel documentation system (Bio-Rad™,USA).

Data analysis

DNA fingerprint data generated by RAPD primers were converted into binary matrix. The presence (1) and absence (0) of each DNA band of a specific molecular weight was recorded for each gel. Pairwise comparisons were made by using the Jaccard similarity coefficient and the NTSYS-PC programme version 2.02 (Rohlf, 1998). Jaccard similarity coefficients were used to construct the unweighted pair-group method with arithmetic means (UPGMA) dendrogram (Jaccard, 1901).

Elution, DNA cloning and sequencing of RAPD fragments

The PCR product (≈1.7 kb) amplified by the RAPD primer OPA 11 from the DNA of *R. solani* isolate RHRG 14 was purified from the gel using Qiagen gel extraction and purification kits (Promega, USA). The cloning of fragments was performed with pGEM-T Easy vector system (Promega, USA) following standard procedures (Sambrook et al., 1989). The competent cells were prepared (Mandel and Higa, 1970) and recombinant plasmid DNA was isolated (Birnboim and Dolly, 1979). The presence of the insert was confirmed by restricting the recombinant DNA with *Eco RI* and colony PCR. The positive clones were selected for sequencing (Xcelris Labs Ltd, Banglore, India).

Designing of SCAR primer

Designing of SCAR marker was done using Primer 3 (v. 0.4.0) software (Rozen and Skaletsky, 2000). From cloned RAPD fragments, one pair of primer was made based on terminal sequences for expression of the selected RAPD marker to a SCAR primer pair SCAR-GS (forward: 5′-GTGGA ACCAA GCATA ACACT GA-3′) and SCAR-GS (reverse: 5′-AGTTT CAACA ACGGA TCTTT GG-3′). During the blast analysis, the SCAR sequences showed more than 98% similarity with 5.8S ribosomal RNA gene, ITS and 28S ribosomal RNA gene partial sequences of *R. solani*. The nucleotide sequences of SCAR were submitted in Genbank (ID: 1616092). PCR was carried out in 25 µL reaction volumes contain-

ing 10 pmol of primer (SCAR-GS F and SCAR-GS R), 1.5 U Taq polymerase, 10X PCR buffer, 3.5 mM $MgCl_2$, and 0.6 mM dNTPs (Bangalore Genei, India).

Amplification was performed in a thermal cycler with the following reaction conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 5 min. Amplified products were separated on 1.4 % agarose gel in 1% TAE buffer, pre-stained with ethidium bromide (1 µg/mL) and electrophoresis was carried out at 70 V for 1 h in TAE buffer. A 100 bp ladder (Bangalore Genei, India) was used as a marker. The gel was observed under ultraviolet light in gel documentation system (Bio-Rad™, USA).

Specificity and sensitivity of PCR method

The DNA of 50 isolates of *R. solani* and four other soil borne pathogens of chickpea, *R. bataticola*, *S. sclerotiorum*, *F. oxysporum* f sp *ciceris* and P. *aphanidermatum* were used to determine the specificity of the markers SCAR GS F and SCAR GS R developed in the present study. To evaluate the sensitivity of the PCR assay, the different concentrations of the genomic DNA obtained from mycelia and infected roots were used to determine sensitivity of the markers. Non template control was also used for comparison. The experiments were repeated at least three times.

Q-PCR assay

Quantitative-PCR (Q-PCR) was performed with a low profile 0.2 ml 8-tube strips without caps, white and optical flat 8 cap strips (Bio-Rad, USA) in the Miniopticon, 48 wells real-time PCR machine from Bio-Rad, USA. Each well contains a 20 µl reaction mixture that includes 10 µl of 1x Sso FAST™ Evergreen master mix (Bio-Rad, USA), 2 µl of primer (5 pmol of each forward and reverse SCAR primer) and 6 µl of sterile double distilled water. Extracted DNA (1µl) at 100 ng/µl was added to the reaction mix. Every DNA sample was analyzed with duplicate real-time PCR reactions. Q-PCR was performed under the following conditions: 95°C for 3 min, 39 cycles of 95°C for 10 s, 60°C annealing for 10 s and 72°C extension for 15 s. Absolute Q-PCR was employed to determine the quantities of *R. solani* DNA in infected chickpea roots. Genomic DNA from *R. solani* with an estimated initial concentration of 100 ng/µl was serially diluted (1:10) with sterile distilled water. The results were analyzed by plotting the log of template concentration against threshold cycle (Ct) values. The extracted DNA from infected plant sample was used as unknown targets for identification and detection of *R. solani*. A real-time PCR assay typically was performed with three replications. The standard error of the mean was calculated accordingly. The sensitivity or minimum detection limit of the assay was estimated so as to quantify and detect the lowest amount of target DNA.

RESULTS

Aggressiveness of the isolates

The isolates (50) of *R. solani* representing 10 states of India (Figure 1) were variable in their aggressiveness on chickpea variety JG 62 and caused 8 to 100% disease incidence (Table 1). Twenty (20) isolates proved to be highly aggressive and caused > 50% disease incidence. Other 20 isolates showed moderate aggressiveness by causing > 20 to 50% disease incidences and the remaining 10 isolates were less aggressive and caused < 20% disease incidence.

RAPD analysis

RAPD analysis generated very distinct banding pattern, which resulted in considerable variability among the isolates collected from different states of India (Figure 1). The number of amplified DNA fragments varied, depending upon the primers and isolates used. In all the chickpea isolates of *R. solani,* the primers used produced reproducible bands with band size ranging from 0.25- 3 kb (Table 2). The primers OPA18 and R28 produced the maximum number of bands whereas; the primers A03 and A08 (Figure 2a) produced the minimum number of bands. OPN 20 also gave 100% polymorphism (Figure 2b). RAPD primers produced 84 polymorphic and one monomorphic (OPA11; Figure 2c) bands. The similarity values of RAPD profiles ranged from 0.52 to 0.93 among all the isolates. A dendrogram (Figure 3) showed estimated similarity from 52 to 93%, reflecting wide range of variability among the diverse collection of the isolates at their molecular level. Based on UPGMA analysis, 50 isolates of *R. solani* were classified into six major groups at 55% of similarity coefficient. Among the six groups, the third and fourth each had 14 isolates belonging to different anastomosis groups (AGs) and geographical locations. The fifth group consisted of two isolates belonging to different AGs from same geographical location (Haryana). The strongest relationship (93% similarity) was scored between *R. solani* isolates RAPG 9 (AG3) and RAPG 11 (AG2-2). The first group consist of three Karnataka isolates [RKNG 9 (AG1), RKNG 10 (AG2-2LP) and RKNG 11 (AG4)], 3 Andhra Pradesh isolates [RAPG 9 (AG2-2), RAPG 9 (AG3) and RAPG13 (AG2-3)] and Gujarat isolate RGJG5 (AG5) were highly diversified in AGs wise. Three isolates from AG5 (RTNG4, RUPG103 and RMPG31) and 2 isolates each one from AG1 (RMHG31) and AG3 (RUPG100) constituted the second groups. The sixth group had eight isolates belonging to different states and AGs.

Development of SCAR marker and Q-PCR

A set of distinctive SCAR marker, namely SCAR GS-F and SCAR GS-R for the identification of WRR of chickpea caused by *R. solani* was developed by cloning and sequencing of the specific DNA fragment (≈1700bp) amplified by RAPD primer OPA11 (Figure 2c). The primer gave a single PCR product of size 285 bp in all the isolates of *R. solani*. The amplification was not obtained in other soil borne plant pathogenic fungi namely, *R. bataticola, S. sclerotiorum, F. oxysporum* f sp *ciceris* and *P. aphanidermatum* (Figure 4). The marker was able to

Figure 1. Map of India showing areas of collection of chickpea isolates of *R. solani*. 1,TamilNadu; 2, Karnataka; 3, Andhra Pradesh; 4, Maharashtra; 5, Gujarat; 6, Madhya Pradesh; 7, Rajasthan; 8, Uttar Pradesh; 9,Delhi; 10, Haryana.

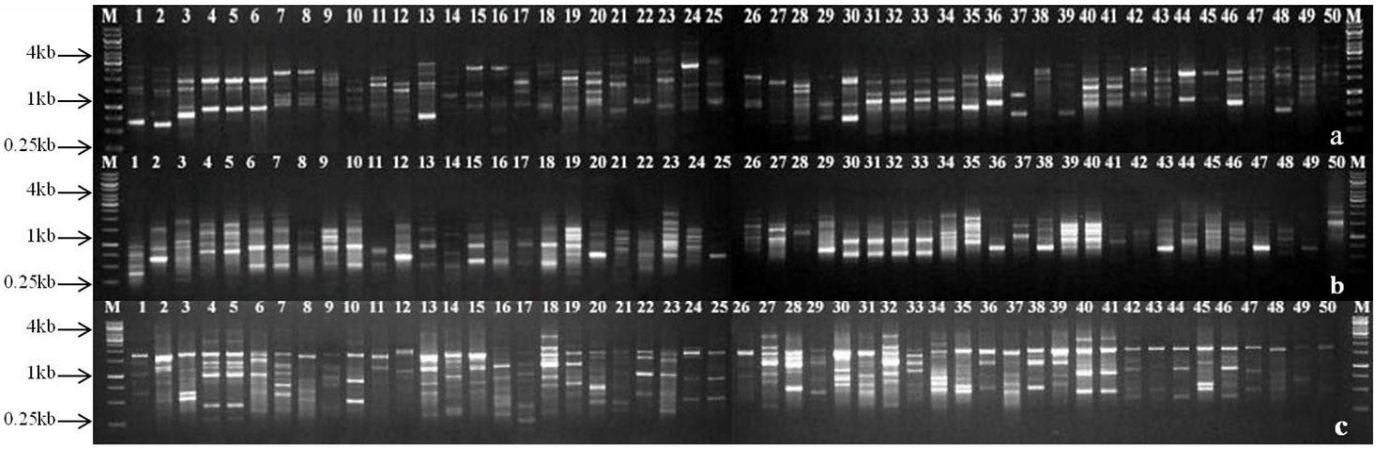

Figure 2. Fingerprint patterns for 50 chickpea isolates of *Rhizoctonia solani* generated by RAPD-PCR with primers A-08 (a), OPN-20 (b), OPA-11 (c). Lanes 1-5, AG 1; 6-7, AG 2-2; 8-10, AG 2-2LP; 11-16, AG 2-3; 17-30, AG 3; 31-34, AG 4; 35-44, AG 5, AG; 45-50 (undetermined AG) and M -1 kb ladder.

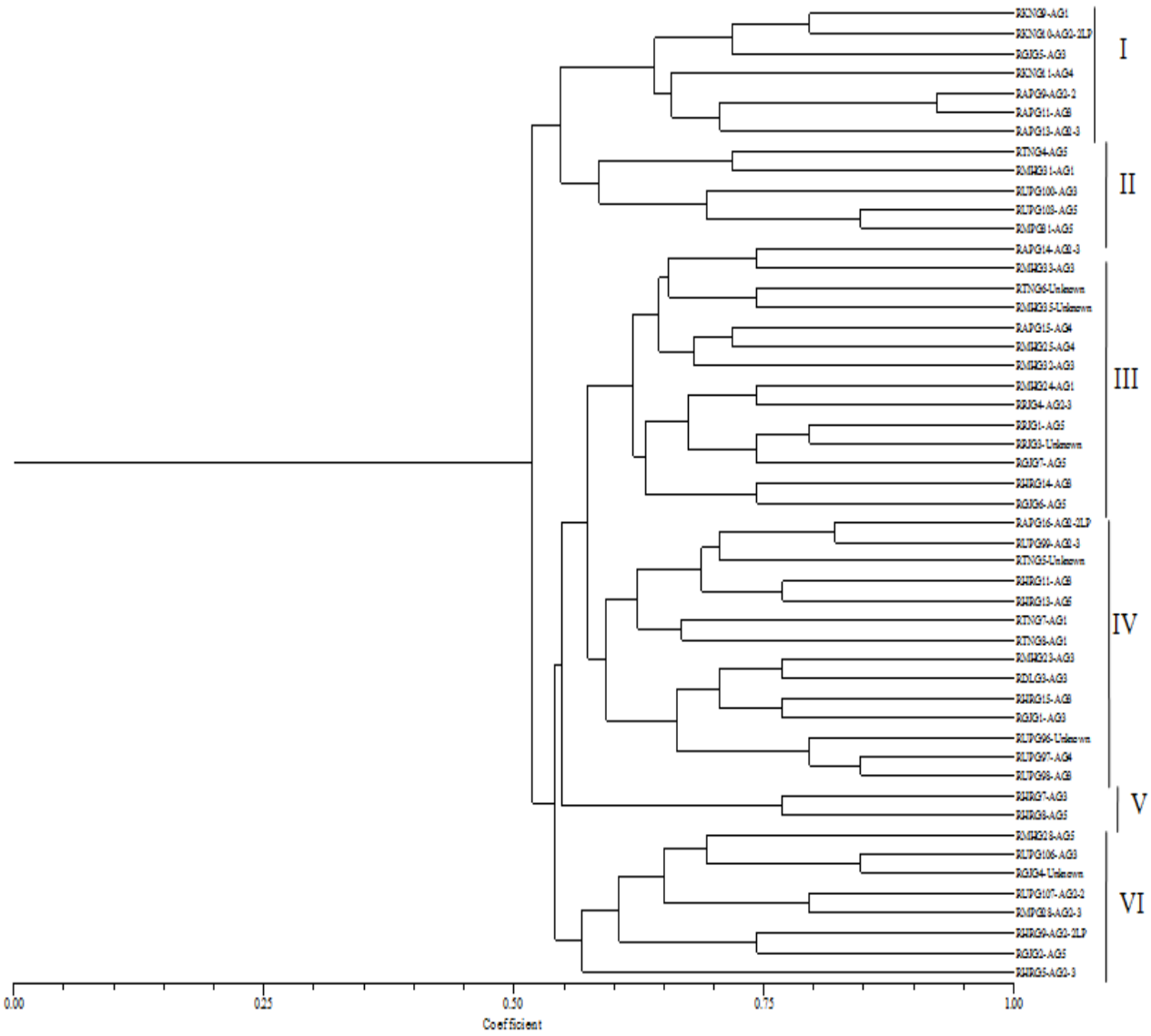

Figure 3. Dendrogram obtained from percentage similarity coefficients after UPGMA-SAHN clustering of band data generated using 10 RAPD primers in 50 isolates of *Rhizoctonia solani* collected from chickpea. The bottom scale represents the percentage of Jaccard's similarity coefficients.

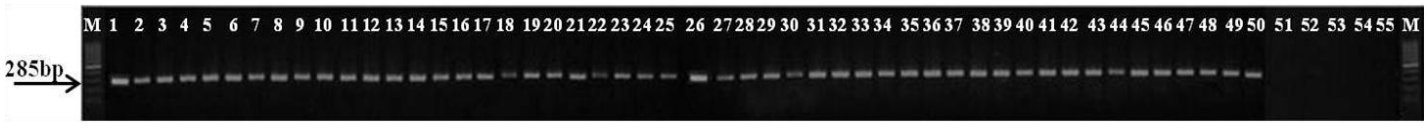

Figure 4. Agarose gel showing 285 bp amplification products from PCR of genomic DNA of *R. solani* using the sequence amplified characterized region (SCAR) primer pair SCAR GSF and R. Lanes 1-5, AG 1; 6-7, AG 2-2; 8-10, AG 2-2LP;11-16, AG 2-3; 17-30, AG 3;31-34, AG 4; 35-44, AG 5, AG 45-50 (undetermined AG), 51, *Rhizoctonia bataticola*; 52*, Sclerotinia sclerotiorum;*, 53, *Fusarium oxysporum* f sp *ciceris*; 54, *Pythium aphanidermatum*; 55, non template control and M-100 bp ladder.

amplify the genomic DNA of *R. solani* upto 0.5 ng concentration. In infected chickpea roots, *R. solani* could be detected by PCR using the SCAR marker with a detection limit of 5ng (Figure 5a and b). The amplification was not obtained from the DNA of healthy chickpea roots. In real time PCR assay, the minimum detection limit of

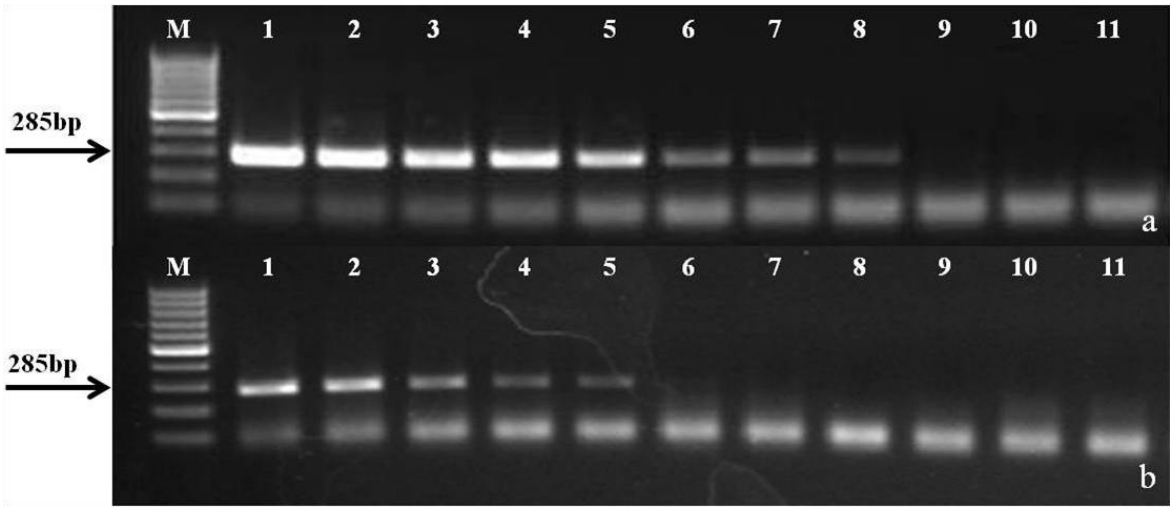

Figure 5. The amplified product obtained with primer SCAR GS F and R from (a) different concentration of genomic DNA of *R. solani* and (b) infected chickpea plants (Lane M, 100 bp ladder; 1, 100 ng, 2, 50 ng; 3, 25 ng, 4, 10 ng; 5, 5 ng; 6, 2 ng; 7,1 ng; 8, 0.5 ng; 9, 0.25 ng; 10, plant genomic DNA; 11, Non template control.

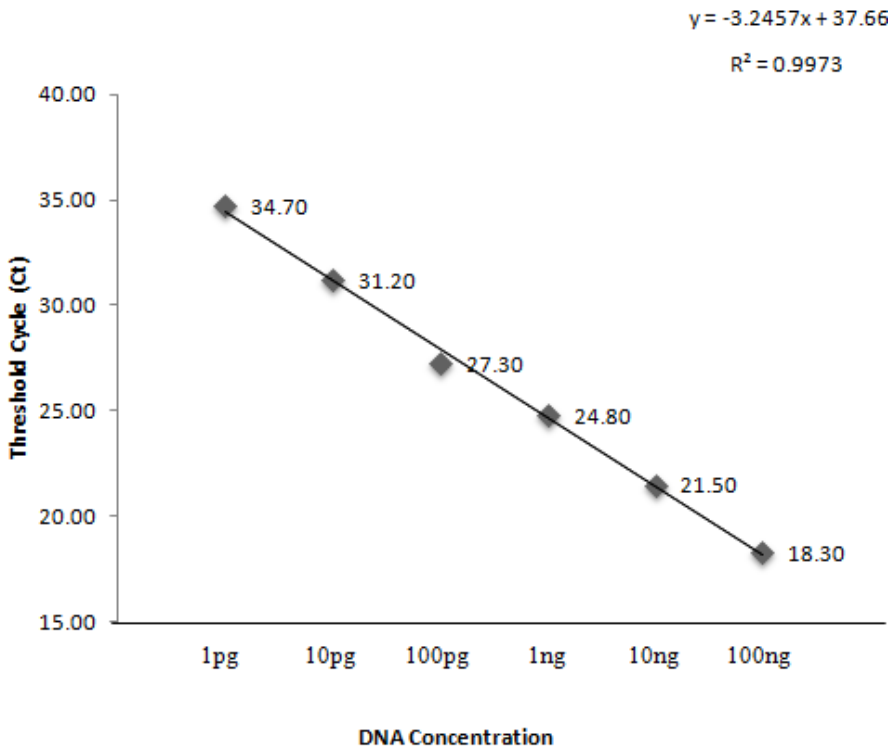

Figure 6. Standard curves generated using Q-PCR for SCAR primer developed for detection and quantification of *Rhizoctonia solani* from infected chickpea plant. A range of DNA concentration from 100ng to 1pg was used to generate the graphs.

SCAR GS-F and R primer was 1 pg at Ct value of 34.70 for infected chickpea plant samples (Figure 6) while the non-template control (NTC) was detected at ct around 37.60.

DISCUSSION

The study clearly indicates that there was considerable variability among *R. solani* isolates collected from different

chickpea growing areas of India. Using RAPD-PCR, closely related strains of a pathogen can be distinguished without prior knowledge of the nature of polymorphic regions. PCR-based DNA fingerprinting, particularly with short oligonucleotide primers, had been used earlier for the analysis of genetic variation in plant pathogens (Purkayastha et al., 2006; Sharma et al., 2005). The isolates of *R. solani* included in the present study showed high genetic variation from 52 to 93%. The RAPD-DNA fingerprint analysis showed variations at the DNA level and these considered suitable for differentiation of *R. solani* isolates (Monga et al*.,* 2004). The chickpea isolates of the pathogen were classified into six groups having the isolates representing different AGs and areas of origin. This evidently indicated the presence of extremely diverse populations of the pathogen in India. A similar result was reported by Dubey et al. (2012) with *R. solani* isolates from different pulse crops. They also observed that the molecular markers were not able to differentiate all the AGs representative isolates into separate groups (Dubey et al*.,* 2012). The isolates were variable in causing wet root rot incidence during pathogenicity test and showed low to high aggressiveness on chickpea variety JG 62. The correlation between aggressive groups and the molecular groups generated through RAPD analysis clearly indicated that out of 50 isolates, 20 isolates were highly aggressive, 20 isolates were medium aggressive while only 10 isolates were less aggressive. The 20 highly aggressive isolates were from seven different states of India representing both northern and southern parts of the country. The majority of the isolates from Tamil Nadu, Gujarat, Rajasthan, Haryana and Delhi were highly aggressive. Each RAPD group had the isolates from different agro-ecological regions, AGs, and aggressive group.

Out of 14 isolates in the RAPD group IV, 10 were highly aggressive. Both the isolates of RAPD group V originnating from Madhya Pradesh were medium aggressive. Thus, RAPD groups were partially corresponding to the aggressive groups of the isolates. The present study also clearly pointed out that most of the isolates were not corresponding to the geographical origin and AGs because the geographical distribution of *R. solani* has been associated with such factors as host range (Anderson, 1982), soil type (Parmeter et al*.*, 1969), altitude (Galindo et al*.*, 1983), and cropping pattern (Ogoshi and Ui, 1983).

The RAPD primers OPA 11 produced a product of ≈1.7 kb which was considered suitable for development of SCAR markers to detection of *R. solani* in chickpea and subsequently a 285 bp size of SCAR marker was developed. The marker was not able to amplify the DNA of the other soil borne plant pathogenic fungi commonly occurring in the chickpea field. The credibility of diagnostic method was based on the lowest detection limit of genomic DNA of the pathogens. The sensitivity analysis of the SCAR markers developed in the present study shows that the PCR with the SCAR markers produced positive results with as low as 0.5 ng template DNA. In infected chickpea, *R. solani* could be detected by PCR using the SCAR primers with a detection limit of 5 ng template DNA. However, a comparison of genomic DNA from fungal culture versus that for DNA from infected samples showed that the plant genomic DNA may reduce the sensitivity of the assay. The marker developed in the present study considered as sensitive and to detect the pathogen in infected chickpea roots. PCR-based SCAR markers were commonly used for detection of several plant pathogens (Larsen et al., 2002; Grosch et al., 2007; Ladhalakshmi et al., 2009; Nithya et al*.*, 2012).

Q-PCR is used as a tool for quick, specific and sensitive detection and quantification of soil borne fungi (Wang et al., 2006; Elsalam et al., 2006; Shishido et al., 2010). In the present study, it was demonstrated that a conventional PCR assay using SCAR-GS F and R primer pair could detect DNA of *R. solani* upto 5 ng but Q-PCR assay achieved minimum detection level upto 1 pg DNA from infected chickpea root samples. Q-PCR is not only accurate in the detection and quantification of plant pathogens but it is also less labor and time consuming technique. This is the first report of molecular detection of *R. solani* in chickpea using a SCAR marker with RT-PCR. In the present study, RAPD analysis established the heterogeneous populations of *R. solani* isolates present in the chickpea growing areas of India. This might be due to the cultivation of different varieties of chickpea having various genetic backgrounds. The information in respect of genetic diversity and AGs distribution of the pathogen generated in the present study could be used for breeding for area specific resistant cultivars of chickpea. The SCAR marker developed could be used successfully to detect *R. solani* causing WRR of chickpea in infected plant samples. The marker may be used for detection of the pathogen from seeds and soils as the pathogen is seed and soil borne in nature.

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Short Communication

Incidence of intestinal nematodes recovered in slaughtered goats in Figuig Province, Morocco

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The main purpose of this study was to investigate helminths incidence in goats. Species recorded were *Trichostrongylus colubriformis, Teladorsagia circumcincta, Skrjabinema ovis, Trichuris ovis, Haemonchus contortus* and *Nematodirus spathiger*, and overall prevalence of helminths was 79% (95/120). The most frequently detected nematodes in the goats were *T. ovis* (62.5%) followed by *T. colubriformis* (52.5%) and *T. circumcincta* (48.33%). The helminth parasites counts in the goats reached the maximum levels in autumn. In relation to sex, the occurrence of helminth parasites was 73.6% in female and 86.6% in male goats.

Key words: Goats, gastro intestinal (GI) nematodes, incidence, seasonal activities, oriental.

INTRODUCTION

Gastrointestinal parasitism is one of the most important diseases of goats, especially nematode infections which are among the major health problems limiting its productivity (Dimander et al., 2000; Johannes et al., 2009). Economic losses are caused by gastrointestinal parasites in a variety of ways. The losses can be through lowered fertility, reduced work capacity, involuntary culling and a reduction in food intake and reduced weight gain, lower milk production, treatment costs and mortality in heavily parasitized animals (Mcleod, 1995; Amadi et al., 2012).

Several studies were done on the incidence of gastrointestinal parasites of goats in different parts of Africa (Katoch et al., 2000; Maichomo et al., 2004; Nwigwe et al., 2013). In Morocco, little information on the distribution and impact of gastro-intestinal tract (GIT) parasitism in these animals is available. And it was therefore important that a survey of helminths of economic importance occurring in this area be conducted. The principal aim of this work was to investigate the incidence of gastrointestinal helminths affecting local goats from oriental zone of Morocco.

MATERIALS AND METHODS

The investigation was carried out in the oriental province of Morocco during the period of November 2005 to May 2006. Animals were selected from different slaughter houses. Immediately after slaughter, the intestines were collected after giving knots on both ends such as at the beginning of the duodenum and ending of the rectum. Then the intestines were brought to the laboratory packed

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Table 1. Prevalence on the basis of sex of the hosts.

Sex	No. examined	No. positive	Prevalence (%)
Female	68	50	73.6
Male	52	45	86.6
Total	120	95	79

Table 2. Prevalence of helminth parasites according to sex.

Parasite name	In female host	In male host	In total host
T. colubriformis	29 (42.64%)	33 (63.46%)	63 (52.5%)
N. spathiger	14 (21.53%)	8 (15.38%)	22 (18.33%)
T. ovis	41 (60.29%)	34 (59.6%)	75 (62.5%)
H. contortus	11 (16.17%)	12 (23.07%)	23 (19.16%)
S. ovis	21 (32.3%)	30 (57.69%)	51 (42.5%)
T. circumcincta	19 (27.94%)	39 (75%)	58 (48.33%)

Table 3. Occurrence of gastrointestinal nematodes in goats in relation to seasons.

Name of parasite	Winter season		Spring season		Autumn season		Summer season		Overall Occurrence (%)
	No. infected (n=35)	Percentage infected	No. infected (n=29)	Percentage infected	No. infected (n=18)	Percentage infected	No. infected (n=13)	Percentage infected	
T. colubriformis	23	65.70	16	20.70	13	72.20	11	84.70	59.65
T. circumcincta	21	60.00	17	58.7	12	66.70	7	53.90	59.90
S. ovis	21	60.00	8	27.60	13	72.20	9	69.20	57.25
T. ovis	7	20.00	6	20.70	7	38.90	8	61.6	35.3
H.contortus	6	17.00	7	24.00	6	33.30	4	30.80	26.27
N. spathiger	6	17.00	8	27.60	6	33.30	4	30.80	27.17
Overall occurrence (%)	39.9		29.9		52.8		44.8		

in a polythene bag as soon as possible. Then, the intestine was cut along the long axis with the help of scissors and the internal mucus membranes were also thoroughly examined, collected parasites were washed several times in normal saline (0.9%) and preserved in luke-warm 70% alcohol (Urguahart and Amour, 1997). Parasites were identified following the keys and descriptions given of Soulsby (1982). The prevalence of each parasite infection was calculated as the number of animals diagnosed positive for a given parasite divided by the total number of animals examined at the particular time (Thrusfield, 2005).

RESULTS AND DISCUSSION

The present study reveals that the overall prevalence of gastrointestinal parasitosis in goats was 79%. The majority of them had multiple helminth parasites. The prevalence of all the nematode infections was found to be significantly higher in male goats than in females (Table 1). The reason for this difference can be attributed to some physiological factor, though both sexes are exposed to similar environmental conditions (Gauly et al., 2006).

A total of sex species of nematodes were recorded and the prevalence of helminthes was higher in males when compared with the females (Table 2). However, most of the researchers have observed higher rate of infection in female hosts when compared with males (Maqsood et al., 1996; Valcarcel and Romero, 1999).

The prevalence of different helminth parasites in relation to season is presented in Table 3, the infection by helminth was observed to be prevalent in all seasons, with some variations. *Trichostrongylus colubriformis, Teladorsagia circumcincta* and *Skrjabinema ovis* were found to be more prevalent in summer and autumn. *Haemonchus contortus* increased in autumn, and declined in winter, higher occurrence was recorded in autumn (52.8%) followed by summer season. our results

are not in agreement with that of Haq (1968) who reported that the highest occurrence was observed in rainy season (95%) followed by winter (90%) and summer (85%). The possible cause of this difference in the percentage of infection could be due to reduced grazing.

According to Kedar et al. (2012), prevalence of gastro intestinal parasites is considerably influenced by the climatic conditions and as far as possible, the evidence of the distribution and prevalence of the diseases is presented by geographical area, roughly corresponding to climatic conditions.

In conclusion, various gastrointestinal parasites have been found in goats in the study area. Hence, the high prevalence rate of helminthiases in livestock needs to be checked periodically. Regular control measure should be practiced and farmers educated in proper use of anthelminthes. It is highly recommended that further studies be done to evaluate the impact of helminth infections on the health and production of small ruminants.

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Short Communication

Anti-bacterial activity of *Coriaria myrtifolia* against *Agrobacterium tumefaciens*: Plant pathogen responsible for crown gall

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The present work aimed to evaluate the antibacterial activity of aqueous and methanolic extracts of *Coriaria myrtifolia*'s leaves against *Agrobacterium* sp. and *Agrobacterium tumefaciens* "plant pathogen responsible for crown gall" in an objective to identify novel antimicrobial agents and to put forward efforts of proving plant's extracts scientific credibility, and determining their spectrum of activity. The bacteria tested were found profoundly sensitive to both of the *C. myrtifolia* extracts. The extent of inhibition was more important by methanolic extract than by aqueous one. The average diameter of inhibition zones ranged from 10.67 to 15.33 mm and 12.68 to 18 mm for aqueous and methanolic extract, respectively. This study was the first to report the antimicrobial activity of extracts obtained from the leaf of *C. myrtifolia* against *Agrobacterium* sp. and *Agrobacterium tumefaciens*. It can be concluded that the observed antibacterial characteristics of *C. myrtifolia* indicate that it might be a promising antimicrobial agent.

Key words: Antibacterial, bacteria, plant extracts, *Coriaria myrtifolia, Agrobacterium tumefaciens.*

INTRODUCTION

Pesticides are an essential input for preventing pre and post harvest crop losses (Saksena, 2001). Synthetic pesticides are commonly used to control phytopathogenic microorganisms (Agrios, 1997). Incessant and extensive use of these synthetic pesticides is inducing serious problems to the life supporting systems due to their residual toxicity (Ferrer and Cabral, 1991; Andrea et al., 2000). It is estimated that only 0.1% of the agro-chemicals used in crop protection hardly reaches the target pest, leaving the remaining 99.9% in the environment, which produce hazards to non target organisms including humans (Pimentel and Levitan, 1986). The large numbers of synthetic pesticides have been banned in the developed countries because of their undesirable attributes such as high and acute toxicity, long degradation periods, accumulation in the food chain and extension of their power to destroy both useful and harmful pests (Barnard et al., 1997; Ortelli et al., 2005).

In-spite of using all available means of plant protection, about 1/3 of the yearly harvest of the world is destroyed by pests and the induced loss is expected to be nearly $300 billion per year (Chandler, 2005). Moreover, many phytopathogenic bacteria have acquired resistance to synthetic pesticides (Williams and Heymann, 1998; White et al., 2002).

Considering the deleterious effects of synthetic pesticides on life supporting systems, there is an urgent need to search alternative approaches for the

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Table 1. Collection sites of the studied plant.

Site	Altitude (m)	North Position	West Position	Exposition	Slope (%)
Bab Berred (BB)	1290	35° 00' 979''	004° 58' 092''	South East	80
Oued el Koub (OK)	140	35° 01' 879''	005° 20' 565''	North	40

management of plant pathogenic microorganisms (Hostettmann and Wolfender, 1997). Green plants represent a reservoir of effective chemotherapeutic agents and can provide valuable sources of natural pesticides (Mahajan and Das, 2003). Biopesticides has been suggested as an effective substitute for chemicals (Verma and Dubey, 1999). Reports are available on the use of several plant by-products, which possess antimicrobial properties, on several pathogenic bacteria and fungi (Dorman and Deans, 2000; Kilani, 2006), but reports are not available on the evaluation of inhibitory action of plants extract on phytopathogenic bacteria particularly in different pathovars of *Agrobacterium* which are known to cause a serious disease at the crown, roots, stems and shoots of many woody and herbaceous plants, causing considerable losses in yield and quality.

Coriaria myrtifolia distributed in the Mediterranean region (France, Italy, Spain, Algeria, Morocco), belongs to the family Coriariaceae and large amounts of tannins from this plant have been used for tanning leather. Hence, the leaves have been used to paint leather in black, and Windholz (1983) reported the use of the fruits in the last century for coloring wine.

The purpose of this study was to evaluate the anti-bacterial activity by the disc diffusion method in the agar medium of the crude extract of *C. myrtifolia* leaves.

MATERIALS AND METHODS

Collection of plant materials

The plant was collected from two sites in Northern Morocco in April 2011, including Bab Berred and Oued el Koub whose geodesic coordinates and characteristics are presented in Table 1. The identification of the plant was made by Professor A. Ennabili from the National Institute of Medicinal and Aromatic Plants, Taounate (Morocco). The leaves of the plant collected were air dried in shadow for a week and then crushed in a mortar. The obtained powder was then used for preparing the two studied extracts.

Preparation of the aqueous extracts

The aqueous extract of *C. myrtifolia* leaves was obtained by decoction of 50 g of plant material in 100 ml of sterile distilled water. At the end of this phase, the extract was filtered under reduced pressure under aseptic conditions and was concentrated in a rotary evaporator until the syrup was obtained which was lyophilized and stored at 4°C. The extract was then used for the antibacterial activity assay.

Preparation of the methanol extracts

The methanolic extract of *C. myrtifolia* was prepared by sonication (30°C; 35 KHZ; 30 min). 45 g of powder was added to 200 ml of methanol. After 45 min of extraction, the mixture (powder and solvent) was filtered and the filtrate was evaporated using a rotary evaporator (90 rpm at 40°C). The final residue was stored at 4°C.

Plant pathogenic bacterial cultures

To test the antimicrobial potential of *C. myrtifolia* powder, two bacterial strains of *Agrobacterium* sp.: (S_7F_3: EF 427851.1 Accesion number et $S_{13}PC_6$: EF 427855.1 Accession number), and one strain of phytopathogenic *Agrobacterium tumefaciens:* (S_7F_3 T: X 67223.1 Accession number) isolated from root nodules of bean (*Phaseolus vulgaris*) (S_7F_3; S_7F_3 T) and chickpea (*Cicer arietinum*) ($S_{13}PC_6$) (Berrada et al., 2012), were used in this test. Well-isolated colonies of each strain were transferred into tubes containing liquid YEM medium and incubated at 28°C for 3 days on a rotary shaker at 160 rpm in order to have a microbial suspension of about 10^6 bacteria/ml.

Anti-bacterial activity assay by the method of disc diffusion

The disc diffusion method was used to determine the antibacterial activities of the extracts against bacterial strains. Mueller Hinton plates were inoculated with 100 µl of the bacterial strain culture (10^6 CFU/ml). Sterile 6 mm diameter filter paper discs were impregnated with 10 µl of the extract and placed onto the inoculated plate (Bauer et al., 1966). Sterile distilled water and methanol were used as negative control while ampicillin (50 µg/ml) was used as positive control. All experiments were performed in triplicate. Plates were then incubated at 28ºC for three days and size of inhibition zone diameters surrounding filter paper disc was measured in mm.

Statistical analysis

Data were expressed as X + SD. X is the diameter of the inhibition zone in mm and SD is the standard deviation. Two factorial ANOVA test was conducted at α = 5% level using the Statgraphics software. Significant differences were determined by multiple range test using 95.0% LSD method.

RESULTS AND DISCUSSION

The antibacterial activity of *C. myrtifolia* extracts is shown in Figure 1. The inhibition zones vary depending on bacterial species and on extract's type. The average diameter of inhibition zones ranged from 13 to 15 mm, 10 to 12 mm, 13 to 18 mm and 12 to 17 mm for *C. myrtifolia* aqueous extracts from Bab berred (Aq. BB) and from Oued El Koub (Aq. OK), methanolic extracts from Bab berred (M. BB) and from Oued El Koub (M. OK), respectively. The largest diameter of inhibition zone was observed for methanolic extracts on the growth of $S_{13}PC_6$ (methanolic extracts of Bab Berred and Oued el Koub)

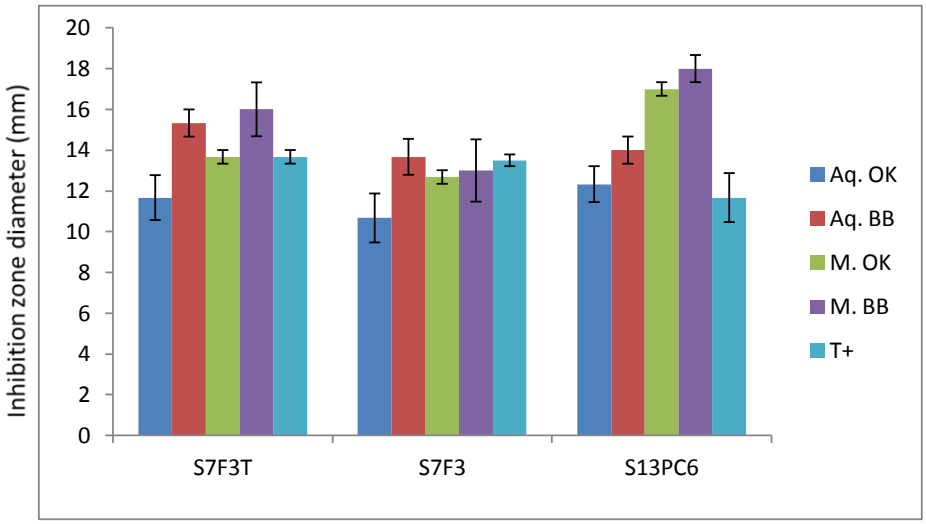

Figure 1. Inhibition zone diameter (in millimetre) of different *Coriaria myrtifolia*'s extracts and antibiotic against the phytopathogenic *Agrobacterium tumefaciens* and *Agrobacterium* sp. Aq.: Aqueous extract, M.: Methanolic extract, B.B.: Bab Berred, O.K.: Oued El Koub, T+: Antibiotic

and S_7F_3T (methanolic extracts of Bab Berred).

The comparison of the inhibition zone diameters for aqueous and methanolic extracts (Figure 1) indicate that methanolic extracts has a greater antibacterial activity in both stations, especially for strains S_7F_3T and $S_{13}PC_6$. This suggests that methanol and water have different solubilising capacity for the bioactive *C. myrtifolia* components which seem to be better extracted with methanol than with water. In general, the activities against test bacterial culture used have shown good activity compared with antibiotic tested. Similar results were found by Boudkhili et al. (2012) for methanolic extracts against some human pathogenic bacteria.

The analysis of variance of the effect of different extracts showed a statistically significant difference in the three strains tested ($P = 0.0127$) and that $S_{13}PC_6$ was the most sensitive to the tested extracts. Moreover, the results (Figure 1) indicate that different extracts of *C. myrtifolia* inhibit significantly ($P = 0.003$) the growth of different strains studied compared to the positive control. The highest inhibition was mostly provided by the methanol extract of Bab Berred followed by methanol extract of Oued el Koub then the aqueous extract of Bab Berred and lastly by the aqueous extract of Oued el Koub. The inhibition activity of the antibiotic (T+) was not significantly different from that of the aqueous extract of Oued el Koub.

These results indicate that under the same operating conditions and for the same strain, the antibacterial activity of *C. myrtifolia* extract from Bab Berred was much greater than that of *C. myrtifolia* extract from Oued el Koub, as well as for aqueous extract or methanolic extract. These differences were statistically significant especially for S_7F_3T and $S_{13}PC_6$. This difference in activity may be due to the influence of environmental conditions on the chemical composition, and hence the concentration of active compounds, of the two origins of this plant. Furthermore, our results show that the antibacterial activity depends on the nature of the solvent used and the bacterial strain tested.

In recent years, field existences of antibiotic resistant phytopathogenic bacteria are increasing (Mandavia et al., 1999). The World Health Organization has banned many pesticides, even if recognized as very important in agriculture, due to their wide range of toxicity to non-target organisms, including humans (Barnard et al., 1997). Some developing countries still use these pesticides despite their harmful effects.

The use of naturally available chemicals from plants, which retards the reproduction of undesirable micro organisms, would be a more realistic and ecologically sound method for plant protection and will have a prominent role in the development of future commercial pesticides (Verma and Dubey, 1999; Gottlieb et al., 2002). Many reports of antibacterial activity of plants extract against human pathogens and their pharma-ceutical application are available (Newman et al., 2000; Gibbons, 2005; Mohana et al., 2008), but not much has been done on the antibacterial activity of plants extract against plant pathogens (Satish et al., 1999). This is mainly due to lack of information on the screening/evaluation of various plants for their antibacterial potential. Thus, the present study reveals that *C. myrtifolia* is a potential candidate that could be successfully exploited for the management of diseases caused by different pathovars of *Agrobacterium*.

Conclusion

In conclusion, this study contributes to the knowledge of the antimicrobial potential of *Coriaria myrtifolia* in vitro. The data presented shows that the plant's extracts studied exerted good antibacterial activity. It appears from this study that:

1) The methanolic extracts of *C. myrtifolia* were more active than the aqueous ones.
2) In the same operating conditions and for the same strains, the antibacterial activity of *C. myrtifolia* extracts from Bab Berred was generally much greater than that of *C. myrtifolia* extracts from Oued el Koub, both for the aqueous and the methanol extracts. This activity is reported for the first time against *Agrobacterium* genus.
3) Whatever the extraction method used, the amount of compounds synthesized in the secondary metabolism of *C. myrtifolia* of Bab Berred is greater than *C. myrtifolia* extracts of Oued el Koub.

Finally, we can say that *Coriaria myrtifolia* of Moroccan origin is an inexhaustible source of natural bioactive substances and compounds. Further studies are needed to focus on the isolation and characterization of biological and chemical bioactive compounds of this important plant, as well to assess their safety.

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