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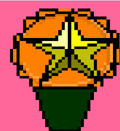

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Departamento de Recursos Naturais e Proteção Ambiental
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Dr. Han-Bo Zhang
Laboratory of Conservation and Utilization for Bio-resources
Key Laboratory for Microbial Resources of the Ministry of Education,
Yunnan University, Kunming 650091.
School of Life Science,
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China

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Dr. Nicole Wolter
National Institute for Communicable Diseases and University of the Witwatersrand,
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South Africa

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Universidade Estadual de Londrina
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Laboratório de Microbiologia Ambiental
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86051-980 Londrina.
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Department of Biochemistry and Microbiology,
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Chulalongkorn University,
Bangkok 10330
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Darjeeling Government College,
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India

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College of Life Sciences,
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Dr. Victor Manuel Fernandes Galhano
CITAB-Centre for Research and Technology of Agro-Environment and Biological Sciences, Integrative Biology and Quality Research Group,
University of Trás-os-Montes and Alto Douro,
Apartado 1013, 5001-801 Vila Real
Portugal

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Instituto de Biotecnologia. Universidad Nacional de Tucuman
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China

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Tehran University of Medical Sciences
Iran

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Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai 90112
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Almeria University
Spain

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Chang Gung memorial Hospital, Chang Gung University
Taiwan

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DDU Gorakhpur University, Gorakhpur-273009 (U.P.)
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South Africa

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Balikesir University
Turkey

Dr. Marija Stankovic
Institute of Molecular Genetics and Genetic Engineering
Republic of Serbia

Dr. Scott Weese
University of Guelph
Dept of Pathobiology, Ontario Veterinary College,
University of Guelph,
Guelph, Ontario, N1G2W1,
Canada

Dr. Sabiha Essack
School of Health Sciences
South African Committee of Health Sciences
University of KwaZulu-Natal
Private Bag X54001
Durban 4000
South Africa

Dr. Hare Krishna
Central Institute for Arid Horticulture,
Beechwal, Bikaner-334 006, Rajasthan,
India

Dr. Anna Mensuali
Dept. of Life Science,
Scuola Superiore
Sant'Anna

Dr. Ghada Sameh Hafez Hassan
Pharmaceutical Chemistry Department,
Faculty of Pharmacy, Mansoura University,
Egypt

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Biochemistry and Molecular Biology
Universidade Federal de Goiás
Brasil

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Public Health & Community Medicine
Faculty of Medicine,
Mansoura University
Egypt

Dr. Hongxiong Guo
STD and HIV/AIDS Control and Prevention,
Jiangsu provincial CDC,
China

Dr. Konstantina Tsaousi
Life and Health Sciences,
School of Biomedical Sciences,
University of Ulster

Dr. Bhavnaben Gowan Gordhan
DST/NRF Centre of Excellence for Biomedical TB Research
University of the Witwatersrand and National Health Laboratory Service
P.O. Box 1038, Johannesburg 2000,
South Africa

Dr. Ernest Kuchar
Pediatric Infectious Diseases,
Wroclaw Medical University,
Wroclaw Teaching Hospital,
Poland

Dr. Hongxiong Guo
STD and HIV/AIDS Control and Prevention,
Jiangsu provincial CDC,
China

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Food Hygiene and Safety, Faculty of Veterinary Science.
University of Extremadura,
Spain

Dr. Jes Gitz Holler
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Aalesund. Central Norway Pharmaceutical Trust
Professor Brochs gt. 6. 7030 Trondheim,
Norway

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College of Life Sciences,
Wuhan University
Wuhan 430072, P.R.China

Dr. Anchalee Tungtrongchitr
Siriraj Dust Mite Center for Services and Research
Department of Parasitology,
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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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African Journal of Microbiology Research

Full Length Research Paper

Pathogenicity of monosporic and polysporic *Bipolaris sorokiniana* isolates to wheat seed and seedling under controlled conditions

Elisandra Minotto[1#], Michele Bertoni Mann[1#], Eduardo Vélez-Martin[2], Thaisa Feltrin[1], Luciana Pasqualini Milagre[1], Cristina Spadari[1], Sueli Teresinha Van Der Sand[1]*

[1]Post-graduation Program in Agricultural and Environmental Microbiology, Instituto de Ciências Básicas da Saúde (ICBS), Universidade Federal do Rio Grande do Sul (UFRGS), Av. Sarmento Leite 500, CEP 90050-170, Porto Alegre, Rio Grande do Sul, Brazil.
[2]Department of Ecology, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves, 9500, Porto Alegre, RS, 91540-000, Brazil.

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***Bipolaris sorokiniana* may present considerable genetic diversity and highly variable pathogenicity and virulence. The pathogenicity of 99 *B. sorokiniana* isolates (27 polysporic and 72 monosporic isolates) from Brazil and other countries was assessed. Based on aborted germination, black point of seed, leaf spot, and coleoptile lesion, the principal component analysis (PCA) was used to evaluate the similarity patterns between isolates considering the variables of pathogenicity. Polysporic isolates presented higher virulence (over 60%), when compared with the monosporic isolates (43%) for all variables, except coleoptile injury. Of all isolates used to infect seeds, 8% were highly virulent, and the score obtained was over 75%, for all variables analyzed. The correlation of *B. sorokiniana* isolates with pathogenicity variables demonstrated that polysporic isolates were more virulent, especially upon seeds, as compared to aerial plant parts.**

Key words: Variability, virulence, *Triticum aestivum* L., spot blotch.

INTRODUCTION

Wheat (*Triticum aestivum* L.) has fundamental importance in humankind's food basis, and today it takes the first place in worldwide agricultural production figures (EMBRAPA, 2013). According to data published by the United Nations Food and Agriculture Organization, global wheat production is expected to reach a record number 708.5 million ton in the 2013 harvest (FAO, 2014). In Brazil, wheat production is about 5,000 to 6,000 ton. The largest cultivated areas, accounting for 90% of the country's production, are in southern (States of Rio Grande do Sul, Santa Catarina and Paraná) and midwest Brazil (States of Mato Grosso do Sul, Goiás and Distrito Federal) (EMBRAPA, 2013).

Wheat is subject to biotic and abiotic limitations, such

*Corresponding author. E-mail: svands@ufrgs.br.

#Authors contributed equally to this work.

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as adverse climatic conditions, soil, pests and diseases. Among the limiting conditions, the phytopathogen *Bipolaris sorokiniana* (Sacc.) Shoemaker,1995; (teleomorph, *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex Dastur) stands out as the causal agent of common root rot, leaf spot, seedling blight and black point in seeds of both wheat and barley cultures, apart from diseases in rye, oat, triticale, sorghum and fescue (Tinline, 1961). However, the most severe symptoms of these diseases are observed in wheat and barley cultures in hot and humid regions, with significant production losses (Kumar et al., 2002).

The pathogenic fungus *B. sorokiniana* uses all plant organs of winter cereals as substrate. For this reason, two distinct disease stages are discernible: the interference in photosynthesis, when the infection occurs in the aerial parts of the plant, and the interference in the search and absorption of water and nutrients, which is the stage that affects underground parts (Forcelini, 1991).

Due to this cosmopolitan nature, spot blotch caused by *B. sorokiniana* is estimated to affect 25 million hectares of wheat plantations worldwide to variable degrees, which accounts for 12% of the total area of the culture that is grown (Duveiller et al., 2005). Seeds infected with the pathogen are highly infectious and stand as the main survival mechanism of this fungus, representing the means by which hot spots of the disease remain active in the field (Forcelini, 1991). *B. sorokiniana* may also play a deleterious role in germination and in the establishment of cereal plantations, leading to aborted seed, seed rot, reducing seed viability, necrosis and discoloration of aerial parts of infected plants (Neergard, 1977).

Spot blotch has symptoms that usually appear on the aerial parts of plants and take the form of oval necrotic lesions surrounded by chlorotic halos. This infection may reduce photosynthetic area and eventually leads to premature plant senescence (Ghazvini and Tekauz, 2007).

Mehta (1978) has recommended the use of combined strategies to control *B. sorokiniana* in wheat and barley cultures. These strategies include the use of resistant varieties, chemical control of soils, waste management, and crop rotation. In spite of the fact that spot blotch is considered one of the most important diseases affecting wheat worldwide, control measures have not produced satisfactory results (Kumar et al., 2007).

In this scenario, the present study assesses the pathogenic potential of monosporic and polysporic *B. sorokiniana* isolates on wheat seeds and seedling under controlled conditions.

MATERIALS AND METHODS

Origin of microorganisms

The fungal polysporic isolates from different regions in Brazil were provided by Empresa Brasileira de Pesquisa Agropecuária- Trigo (EMBRAPA- Trigo, Passo Fundo, Brazil), while the other isolates used in this study were kindly provided by the International Maize and Wheat Improvement Center (CIMMYT, México). All isolates used were obtained from seeds and tissues of wheat plants. The biological material was deposited in the collection of the Environmental Mycology Laboratory, DMIP, ICBS, UFRGS. Ninety-nine *B. sorokiniana* isolates, characterized morphologically, physiologically and molecularly (Müller et al., 2005; Poloni et al., 2008; Nascimento and Van Der Sand, 2008; Mann, 2014; Mann et al., 2014) were used in the pathogenicity assay, of which 27 were polysporic and 72 were monosporic isolates (Table 1).

Table 1. Origin of *B. sorokiniana* isolates from different regions in Brazil and other countries.

Isolates code	Origin
98004 P, A, B, C	Cruz Alta, RS, Brazil
98007 P, A, C	Cruz Alta, RS, Brazil
98030 P, A, C	Cruz Alta, RS, Brazil
98032 P, A, B, C	EngenheiroBeltrão, PR, Brazil
CEV53 A, B	Guarapuava, PR, Brazil
98011 P, A, C	LagoaVermelha, RS, Brazil
98012 P, A, C	LagoaVermelha, RS, Brazil
98031 P, A, B, C	Nova Estância, PR, Brazil
98028 P	Pelotas, RS, Brazil
98025 P, A, C	Piratini, RS, Brazil
98026 P, B, C	Piratini, RS, Brazil
98042 P, A, B, C	Piratini, PR, Brazil
1992 B, C	Planaltina, GO, Brazil
98010 P, A, B, C	Santa Rosa, RS, Brazil
98041 P, A, B, C	União da Vitória, PR,Brazil
98023 P, A, B, C	União da Vitória, PR,Brazil
98013 P, A, B, C	União da Vitória, PR,Brazil
98017 A, B, C	Samambaia, PR, Brazil
CEV48 P, A, B, C	Tapera, RS, Brazil
98034 P, A, B, C	Unknown
NRRL5851 P, A, B, C	South Africa
CFO201 P, A, B, C	South Africa
A20 P, A, B, C	Canada
1965 P	Copenhagen, Denmark
BS15M2 P, A, B, C	Delicias, Chihuahua, Mexico
BS16M1 P, C	Delícias, Chihuahua, Mexico
BS18M2 P, A, B, C	Poza Rica,Vera Cruz, Mexico
CMO105 P, A, B, C	Mexico
BS52M1 P, A, B,	Monterrey, Nuevo Leon
CS1004 P, A, B, C	Hanoi, Vietnam

P: Polysporic *B. sorokiniana* isolate; A, B, and C: Monosporic *B. sorokiniana* isolate originated from the respective polysporic isolate; CEV: *B. sorokiniana* isolate from barley.

Monosporic and polysporic cultures

The monosporic cultures were obtained from the aerial mycelia of the polysporic cultures grown on plates with potato dextrose agar (PDA). A 0.85% saline solution was poured over the plated colonies, and the conidia were transferred to microcentrifuge tubes.

The contents of tubes were homogenized thoroughly to guarantee complete conidia release. The suspension was transferred to a Petri dish with PDA and incubated at room temperature for 2 h. Using a stereomicroscope with optical magnification of 40×, the conidia were transferred using plates with PDA. The plates were maintained at 24 ±2°C until the complete colonies developed, and then were stored at 4°C. Each spore culture was identified with a letter (A, B and C).

Fungal inoculum preparation

B. sorokiniana isolates were multiplied on a culture medium prepared with vegetable broth and carrot agar specific for sporulation and incubated in a BOD stove for 10 to 15 days at 25ºC in a 12-h photoperiod. To standardize the fungal inoculum, 5 mL sterile saline (0.85%) containing Tween 80 (0.1%) were added to colonies. Then, colonies were lightly streaked using a Drigalski spatula, spores were removed, and the suspension was transferred to sterile glass test tubes. Final spore concentration was adjusted to 10^6 spores/mL by counting conidia in a Neubauer chamber.

Pathogenicity assay

The pathogenicity assay was carried out using the 99 *B. sorokiniana* isolates. Samples of 100 wheat seeds, cultivar BRS Buriti, which is considered moderately susceptible to leaf spot, were disinfected using ethanol 70% for 2 min, sodium hypochlorite 2.5% for 2 min, and three wash runs with sterile distilled water. Samples were then placed in tubes containing the previously adjusted spore suspension and left at room temperature for 24 h. After, seeds were incubated according to a modified version of the Blotter test method. Each 100-seed sample was divided in groups of 25 seeds that were placed one by one on wet filter paper sheets, with four repeats. The sheets containing seeds were folded as a sachet, which was incubated in a seed germinator (JP-1000, J. Prolab) at 25°C in a 12-h photoperiod for 10 days. After incubation, wheat seeds and seedlings were individually assessed for aborted germination, black point of seed coleoptile lesion and leaf spot. The assay was carried out in10 blocks, each of which included a control group of seeds that were not challenged with *B. sorokiniana*. After the lesions were analyzed, all the tissues of the organs were submitted to re-isolation of the phytopathogen using culture conditions on PDA plates. Then, growth analysis of the structures under the microscope was carried out.

Statistical analysis

A descriptive statistics of the virulence of *B. sorokiniana,* was carried out for the four variables, expressed as percent values: for aborted germination, black point of seed, leaf spot, coleoptile lesion. This evaluation was visually determined by the presence or absence of symptoms.

The differences in pathogenicity between controls and groups of monosporic and polysporic isolates were assessed using the one-factor analysis of variance followed by an analysis of the differences between treatments using the randomization test, as described by Pillar and Orlóci (1996).

The differences in pathogenicity patterns between isolates based on the four variables assessed (aborted germination, black point of seed coleoptile lesion and leaf spot) was evaluated using the principal component analysis (PCA) (Person, 1901).

The statistical analyses were carried out using the application R (R Development Core Team, 2008) and the action interface for Excel (Estatcamp, 2013). Normality of variables was tested using the Shapiro-Wilk test. The analyses of variance and multivariate analyses were made in the MULTIV (Pillar, 1997).

RESULTS

Results of the four variables assessed indicated that the monosporic and polysporic isolates of *B. sorokiniana* strongly induced the diseases in wheat seed and seedlings, as compared to controls (Table 2).

The comparison between treatment groups (monosporic, polysporic and control isolates) revealed a significant difference between monosporic and polysporic isolates for aborted germination, black point of seed, leaf spot. The exception was observed for coleoptile lesion (Table 2).

The monosporic isolates virulence on germination, showed a median of 59.5 seeds with aborted germination, and the variation ranged from a minimum of 16 to a maximum of 100 seeds. The virulence of polysporic isolates had a higher median, of 75 seeds with aborted germination, and the variation ranged from a minimum of 43 to maximum of 110 seeds (Table 2 and Figure 1).

The highest virulence towards germination (values above the third quarter) was exerted by 18 monosporic isolates, with values over 73.25% for aborted germination, and by 6 polysporic isolates, with aborted germination values over 84% (Table 2 and Figure 1).

The virulence of *B. sorokiniana* isolates on wheat seeds led to high deterioration, with a median value of 100 seeds with black point, both for monosporic and polysporic cultures. The data analyses showed statistically significant differences between all treatments. Mean number of seeds affected by black point was higher after treatment with polysporic cultures (97.4% ±8.1), followed by seeds treated with monosporic cultures (83.9% ± 27.0) and controls (13.2%) (Table 2). On the other hand, 5.4% of monosporic isolates did not cause symptoms in seeds, and did not differ from controls. Allpolysporic isolates caused black point of seeds, with the lowest value of 61% of seeds.

Coleoptile lesion caused by polysporic and monosporic *B. sorokiniana* isolates presented medians of 82.0 ±34.1 and 67.9 ± 34.3, respectively (between zero and 100%). The analysis of variance, to compare groups, indicated a significant difference between the control and the treatment groups, though this difference was not observed between monosporic and polysporic isolates (Table 2 and Figure 1).

Incidence of lesions on leaf blades presented medians of 44.6 and 65.4 for monosporic and polysporic isolates, respectively (Table 2 and Figure 1). Among the most virulent isolates that caused leaf spot (above the third quarter), 25% were monosporic isolates, causing the effect in more than 69% of leaves, while 22.2% of polysporic isolates triggered the effect in more than 79.8% of leaves.

Analysis of the virulence of monosporic and polysporic isolates

The similarity pattern in the virulence of the isolates,

Table 2. Analysis of variance between the treatment groups of monosporic and polysporic isolates and control, for the variables: seed aborted germination, black point of seed, leaf spot and coleoptile lesion.

Variable	Treatment	N	Mean	median	Min	Max	SD	Lower limit (LL mean)	Upper limit (UL mean)
Aborted germination (1)	C[a]	11	25.7	24	16	41	7.7	20.6	30.9
	M[b]	74	60.7	59.5	16	100	19.9	56.1	65.3
	P[c]	27	72.4	75	43	100	17.5	65.5	79.3
Black point of seed (2)	C[a]	11	13.2	10	0	44	16.1	2.3	24
	M[b]	74	83.9	100	0	100	27.0	77.6	90.1
	P[c]	27	97.4	100	61	100	8.1	94.2	100
Coleoptile lesion (3)	C[a]	11	9.3	0	0	83.3	24.8	0	26
	M[b]	72	60.7	67.9	0	100	34.3	52.6	68.8
	P[b]	26	69	82	0	100	34.1	55.3	82.8
Leaf spot (4)	C[a]	11	5.6	0	0	54.5	16.3	0	16.6
	M[b]	72	43	44.6	0	100	33.7	35.1	51
	P[c]	26	59.9	65.4	0	100	31.3	47.3	72.6

C: Control, M: Monosporic isolate, P: Polysporic isolate, N: Number of treatments, Min: Minimum seeds, Max: Maximum seeds, SD: Standard deviation, CI: Confidence interval. Groups followed by different letters differ significantly from one another based on the probabilities obtained by the pairwise randomization test: (1) P= 0.001 for C-M. P= 0.001 for C-P and P= 0.01 for M-P; (2) P= 0.001 for C-M. P= 0.001 for C-P and P=0.009 for M-P; (3) P= 0.005 for C-M. P= 0.001 for C-P and P= 0.02 for M-P; (4) P= 0.001 for C-M. P= 0.001 for C-P and P= 0.296 for M-P.

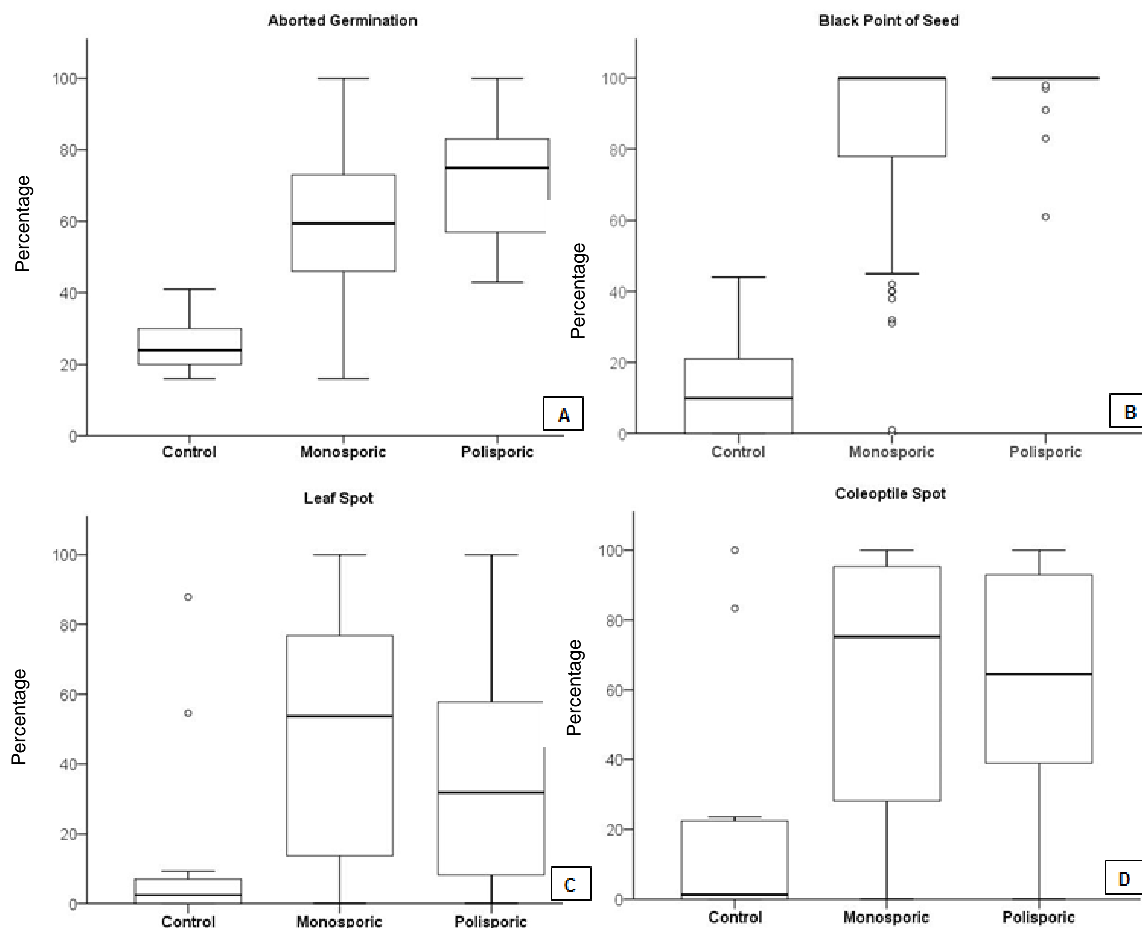

Figure 1. Boxplot of the variables: aborted germination, black point of seed, leaf spot, and coleoptile lesion in the pathogenicity test of the monosporic and polysporic isolates of *B. sorokiniana*.

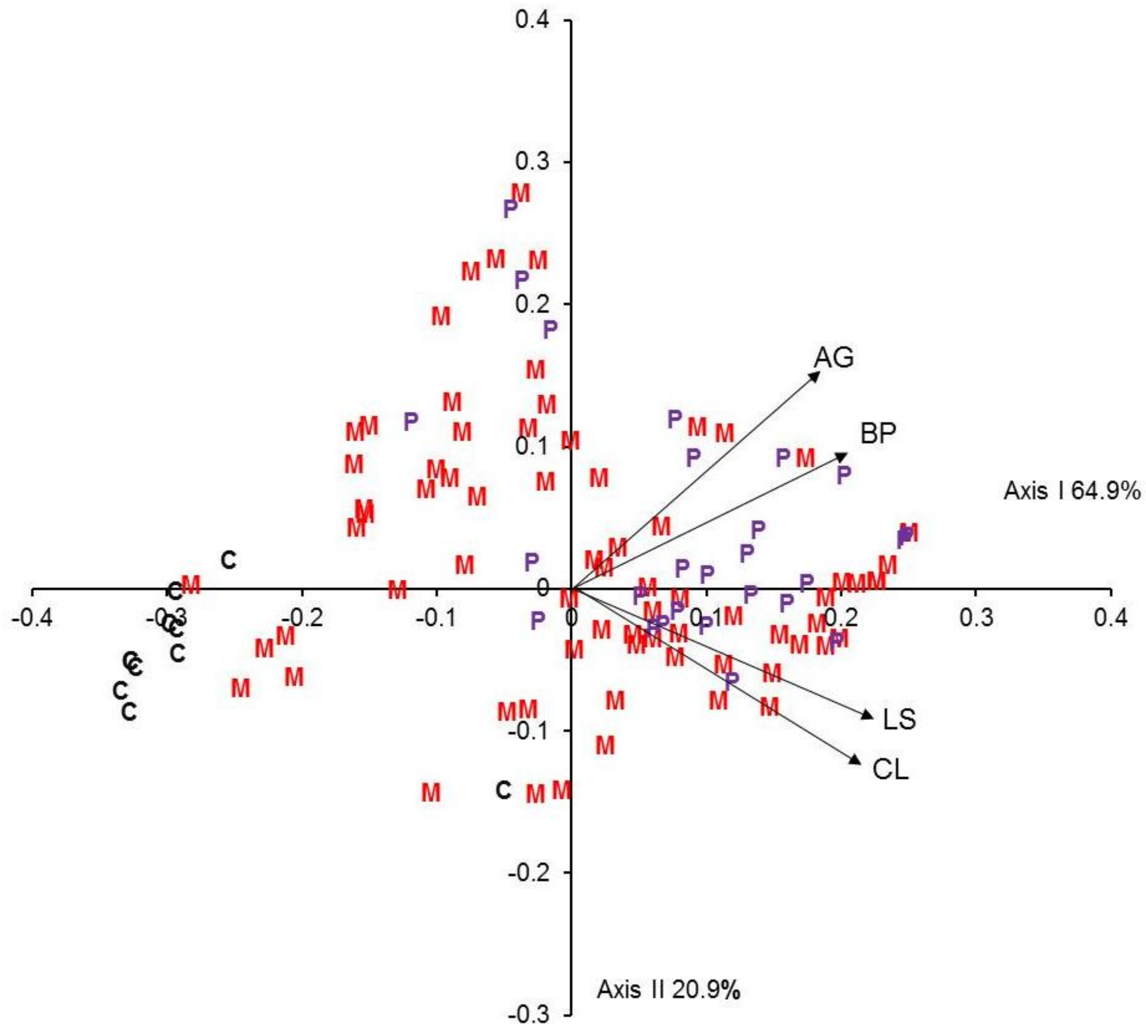

Figure 2. Ordination chart for *B. sorokiniana* isolates in terms of pathogenicity variables constructed based on the principal components analysis and correlation as a measure of similarity between the variables: (A) aborted germination (AG); (B) black point of seed (BP); (C) leaf spot (LS); (D) coleoptile lesion (CL). The percentage of variation in each axis and the variables that are correlated with at least one of the two axes is indicated. C: Control, M: Monosporic isolate, P: Polysporic isolate.

based on the four variables evaluated, simultaneously is shown in Figures 2 and 3. Ordination axis I contains 64.9% of the total variation of pathogenicity data, in which all variables exhibited high, positive correlation (aborted germination = 0.71, black point = 0.79, leaf spot = 0.87 and coleoptile lesion = 0.83) with this axis and the major contribution was that of leaf spot with 29.5% of the total variation in this axis. The position of isolates on axis I allows identifying the most virulent isolates, on the right, namely CEV48P, 98042P, 98042C and CFO201B. Low pathogenicity isolates are at the far end of the axis, especially 98012C, 98023B, 98026C, CFO201A and CS1004A.

Axis II contains 20.9% of the total variation in pathogenicity data and allows differentiating the most pathogenic isolates to wheat seeds from the most pathogenic to the aerial parts of the plant. Aborted germination and black point of seed had positive correlation with axis II (0.58 and 0.36, respectively), while leaf spot and coleoptiles lesion presented negative correlation (-0.35 and -0.48 respectively). The highest contribution was given by aborted germination, with 40.9%.

The ordination chart of isolates also reveals a clear distinction between control and monosporic and polysporic isolates (Figure 2). Also, monosporic isolates presented higher variation in virulence, as compared to polysporic isolates. However, these were a little more specialized in terms of pathogenicity, affecting more seeds than the aerial parts.

Of the isolates used to infect seeds, 8% presented the highest virulence, with virulence of over 75% for all

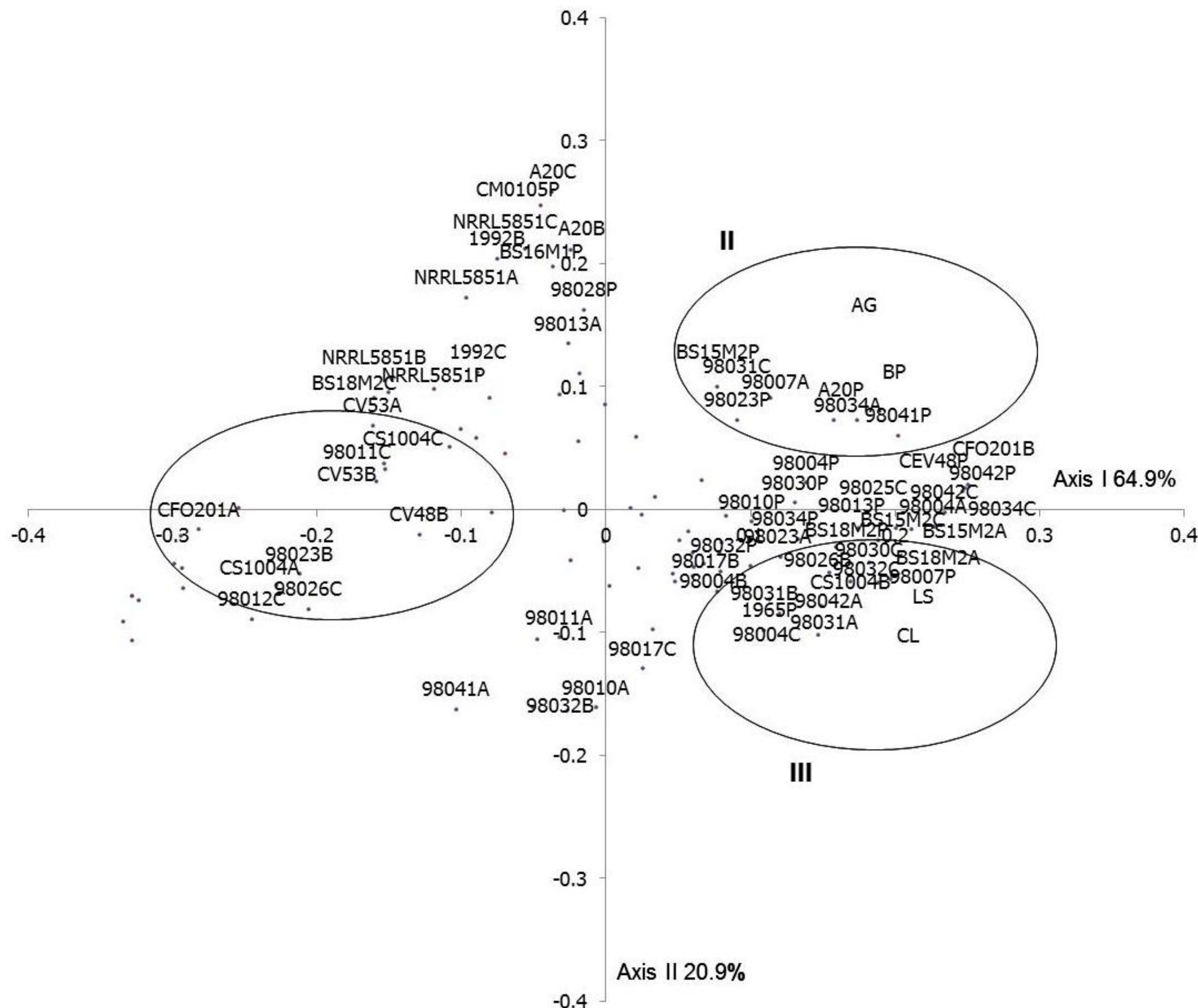

Figure 3. Ordination chart for *B. sorokiniana* isolates in terms of pathogenicity variables constructed based on the principal components analysis and correlation as a measure of similarity between the variables aborted germination (AG), black point of seed (BP), leaf spot (LS), and coleoptile lesion (CL). The identification codes of isolates with high and low values are shown in axes I and II. Isolates presenting intermediate values were not labeled, and are identified by dots. The ellipsis I indicates isolates with low pathogenicity, ellipsis II signals highly pathogenic isolates affecting mainly seeds, and ellipsis III indicates highly pathogenic isolates affecting mainly aerial parts.

variables assessed. The isolates with the highest and lowest scores in axis I and II were the most virulent when the four variables are considered as a set, 98004A, 98025C, 98034C, 98042P, 98042C, CEV48P and BS15M2A (Figure 3). Black point of seed was observed in all monosporic and polysporic isolates, except for isolates 98031P, 98012C, 98041A and CFO201A. On the other hand, monosporic isolates 98012C, 98041A and CFO201A presented low virulence, with symptoms observed in less than 1% of wheat seedlings and seeds.

DISCUSSION

In general, microorganisms present high genetic diversity, leading to differences in morphology, physiology and pathogenicity. Variations in the use of substrates, tolerance to determined temperature and pH ranges, production of toxins and other metabolites are among the manifestations of physiological distinctions in one population, which often result in variation of pathogenicity of biotypes (Machado, 1980).

The presence of symptoms and the wide variation in this pathogen's virulence patterns are reported based on the analysis of pathogenicity variables, which indicate that polysporic isolates are more virulent to leaves, with values over 60%, when compared with monosporic isolates, with values over 43% (Table 2). The monosporic cultures were used to reduce the effect of heterokaryosis, since one single conidium may be homokaryotic or present reduced variability, which makes it easier to identify pathotypes based on isolate virulence. This

characteristic may be linked with the different genes present in heterokaryotic cells of monosporic isolates, which in turn may manifest in different ways, depending on the quality and quantity of nuclei contained in cells and on the roles played by the environment and the host (Tinline, 1961). This may explain the wider spectrum of monosporic isolates on the pathogenicity variables shown in Figure 2.

Pathogenicity tests carried out by Christensen (1925) using 37 monosporic *H. sativum* isolates indicated that 18 formed zones in BDA medium, which differed from the parental colony as compared to morphology and pathogenicity. In a previous study, the virulence, morphology and growth rate in culture medium of 10 *B. sorokiniana* isolates from different regions in Brazil were analyzed in wheat. Wide variations in morphology and growth rates were observed between parental and re-isolated isolates. However, no relationship between morphological variability and virulence was detected between these two types of isolates (Oliveira et al., 1998) or origin of isolates (Valim-Labres et al., 1997).

The results obtained in the present study show that polysporic isolates exerted higher pathogenic action, predominantly in seeds, as compared to aerial parts. Often the pathogens that cause common root rot also cause different diseases in one single plant species; however, most specific symptoms in one plant are regulated by infection time and soil conditions, mainly temperature and humidity (Wheeler and Rush, 2001). Our results also reveal that polysporic isolates, which presented high pathogenicity levels in seed germination, did not show the same virulence indices in comparison with the respective monosporic isolates. For example, isolate 98041P inhibited germination in 97% of wheat seeds, while the monosporic isolates generated from the same polysporic strain reduced germination by approximately 50%. According to Mehta (1998), a likely source of variability may be inherent to the fungus itself, since its pathogenicity may vary with time.

Duveiller and García Altamirano (2000) showed that *B. sorokiniana* isolates from different parts of a plant did not cluster according to virulence, when they were reinoculated. In this sense, the authors discovered that the number of leaf spot varies with the isolate used for inoculation, and that this isolate does not depend on the organ from where it was isolated (Duvellier and García Altamirano, 2000). Fetch and Steffenson (1999) observed variation in virulence patterns of *Cochliobolus sativus* in relation to barley cultivars and to the development stage of plants.

In the present study, we observed that the most severe symptoms were associated with germination and black point of seed, with reduced germination and high levels of rot (Table 2). This condition is mainly due to the hemibiotrophic nature of *B. sorokiniana* and to the complex enzymatic apparatus it has, which is able to use any organ of a plant as nutritional substrate. The seed is considered one of the most efficient means of transmission and dissemination of phytopathogenic agents, especially overlong distances. In unaffected areas, seeds mediate the introduction of pathogens, which may be spread, selected and distributed by means of primary disease hotspots (Maffia et al., 1988).

The incidence of *B. sorokiniana* in wheat seeds is often observed negatively, affecting germination and triggering the occurrence of symptoms in plants and seeds, and even causing the death of plants (Lasca et al., 1983). The association of the pathogen to seeds is an efficient mechanism of survival and dissemination, and is the main reason behind the outbreaks of epidemics in wheat production regions in Brazil (Goulart et al., 1993). According to Kumar et al. (2002), infections may be so severe that the infected plants wither, without producing one single seed. Under conditions that favor the pathogen's life cycle, spikelets may be affected, causing seeds to dry out.

The pathogenicity assays were carried out using the wheat cultivar BRS Buriti, which is moderately susceptible to leaf spot and is recommended for the establishment of wheat plantations in winter, in southern Brazil. In this sense, the use of *B. sorokiniana* isolates from different regions of Brazil and the world may indicate that pathogenicity levels differ, which in fact was not observed. The results obtained did not afford to group *B. sorokiniana* isolates by geographic origin or the definition of similarity patterns in pathogenic action. Maraite et al. (1998) analyzed 360 wheat leaf samples from 10 countries presenting symptoms of the disease, and did not observe specific relationships between pathogenicity in terms of geographic origin and genotype.

Conclusion

The most interesting aspects observed in the present study are associated with the wide pathogenic variability of *B. sorokiniana* isolates. Virulence based on conidial origin were established using the correlation between monosporic and polysporic isolates for the variables, aborted germination, black point of seed, leaf spot and coleoptile lesion. Polysporic isolates presented higher virulence (over 60%), when compared with monosporic isolates (43%) for all variables, except coleoptile lesion, and increased aggressiveness was observed against the seed.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Phytochemical profile and antioxidant and antimicrobial activities of hydroethanolic extracts of *Ficus pumila*

Natália Maria Noronha[1], Grazielle Esteves Ribeiro[2], Ingridy Simone Ribeiro[1]*, Marcos José Marques[1], Luiz Felipe Leomil Coelho[2] and Jorge Kleber Chavasco[1]

[1]Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal de Alfenas (UNIFAL-MG), Brazil.
[2]Programa de Pós-Graduação em Biociências Aplicadas À Saúde da Universidade Federal de Alfenas (UNIFAL-MG), Brazil.

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Natural products, mainly of plant origin are widely used in popular medicine. This study aimed to evaluate the therapeutic potential by examining the hydroethanolic extracts of fresh and dried leaf, stem, root and fruit of *Ficus pumila* concerning their phytochemistry profile and antimicrobial, antioxidant and cytotoxic activities. The results show the presence of tannins and flavonoids in all extracts. The dried root extract exhibited the highest content of phenolic compounds (724.39 mg GA/g) and the dried leaf showed the highest content of flavonoids (15.30 mg quercetin/g). The extracts showed activity against *Bacillus subtilis*, *Bacillus cereus*, *Micrococcus luteus*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Proteus mirabilis*. None of the extracts showed anti mycobacterial or antifungal activity. The highest antioxidant activity by scavenging of free radical DPPH was exhibited by the extract of fresh stem (EC_{50} = 12.81 µg/mL) and only the fresh leaf extract showed cytotoxicity with CC50 at 5 mg/mL.

Key words: *Ficus pumila*, antioxidant activity, antimicrobial activity, phenolics, flavonoids, cytotoxicity.

INTRODUCTION

During the last decades, the development of efficient drugs to combat microbial infections has revolutionized medical treatment, leading to drastic reduction in mortality from these diseases. Moreover, the widespread use of antibiotics unfortunately made the microorganisms develop resistance to antimicrobial agents, including drugs used to treat tuberculosis (Silveira et al., 2006).

Microbial resistance is considered a major global public health problem because it imposes restrictions for the treatment of infections. Thus, the increase of multi-resistant strains to antimicrobial drugs available in the market has led to search for new antimicrobial agents (Oplustil, 2012).

Natural products, especially of plant origin, are an excellent source for finding new antimicrobial molecules as the natural compounds to countain a much higher molecular diversity to those derived from synthetic products. For a long time, numerous plants have been used in the treatment of several diseases. Thus, research focused on the study and evaluation of plants can lead to

*Corresponding author. E-mail: ingridyribeiro@gmail.com. Tel: +55.35.32991475.

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discovery of new drugs, especially those with antimicrobial activity (Novais et al., 2003).

Ficus pumila, known as Ivy-girl or cat's nail, is an ornamental Moraceae family plant, native from Japan, China and Australia, which has been widely used in traditional Chinese medicine, as having analgesic and anti inflammatory activities. Although it has several biological activities, there are still few reports of its antioxidant and antimicrobial activities (Liao et al., 2012; Lorenzi and Hermes, 2001).

In this context, this study aimed to analyze the chemical composition, antimicrobial and antioxidant activities of hydroethanolic extracts of root, stem, leaves and dried and fresh fruits of *F. pumila* as a source of possible new agents to be used in the treatment of infections caused by Gram-positive and Gram-negative bacteria, mycobacteria and yeasts.

MATERIALS AND METHODS

Collection and identification of *F. pumila*

Sample collection of root, stem, leaf and fruit of *F. pumila* were performed at the Airport Garden District in Alfenas/MG (21º 27'50.70"S; 45º 56'32.28'' W), to a rise of 908 m, in November 2012. The plant exsicata was deposited and identified at Federal University of Alfenas (MG, Brazil) receiving the registration number 2339.

Obtaining the extracts

Hydroethanolic fresh plant extracts were prepared and dried in a proportion of 20% (w/v) using ethanol 70% (v/v). Prior to extraction, the samples of root, stem, leaf and fruit of the plant were washed in running water and manually cut into smaller pieces with the help of a knife. For the preparation of the dried extracts, the plant samples were dried, crushed and subjected to the analysis of particle size.

The extracts were prepared from fresh and dried plant by maceration. After extraction, all extracts were filtered in filter paper. Then, they were concentrated by rotaevaporator apparatus using negative pressure of 500 mm Hg at a temperature of 60ºC, frozen and lyophilized (Silva et al., 2010).

The evaluation of the phytochemical profile of extracts

Phytochemical screening tests based on colorimetry and precipitation for detection of anthraquinones, flavonoids, tannins, saponins and alkaloids were performed (Costa, 1994). The total phenolic content was measured from an aliquot of 0.5 mL each extract at 0.1 mg/mL and mixed with 2.5 mL of Folin-Ciocalteau reagent (diluted 1:10 in distilled water) plus 2.0 mL of Na_2CO_3 4% (w/v) in distilled water.

After 2 h of incubation and being protected from light at room temperature, the absorbance was measured at 750 nm in a spectrophotometer. The results were expressed as milligrams of gallic acid equivalents per gram of sample (mg GA/g) and they were calculated by using a calibration curve ranging from 5 to 100 µg/mL of gallic acid (Singleton et al., 1999).

The content of flavonoids was measured in an aliquot of 0.5 mL of each extract (at a concentration of 1.5 mg/mL) and mixed with 1.5 mL of ethanol, 0.1 mL of aluminum chloride ($AlCl_3.6H_2O$) 10% (w/v), 0.1 mL 1 M potassium acetate plus 2.8 mL of distilled water, and 5 mL of final volume. After 30 min, the absorbance of the mixture was measured at 425 nm. The total flavonoid standard curve was made using a quercetinstandard solution. The amount of flavonoids was expressed as milligrams of quercetin equivalents per gram of sample (mg quercetin/g), and the values were shown as mean of triplicate determinations (Kalia et al., 2008).

The microbial strains used

For microbiological evaluation. the following microorganisms were used: a) Gram positive bacteria: *B. cereus (ATCC 11778)*, *B. subtilis (*ATCC 6633), *M. luteus (*ATCC *9341)*, *E. faecalis* (ATCC51299), *S. aureus (*ATCC *6538)*;b) Gram negative bacteria: *Escherichia coli (*ATCC8739*)*, *Enterobacter aerogenes* (LMI-UNIFAL), *Proteus mirabilis (*ATCC*25922)*, *Pseudomonas aeruginosa (*ATCC *9027)*, *Salmonella typhimurium (*ATCC *14028)*,*Serratia marcescens (LMI-UNIFAL)*; c) Mycobacteria: *Mycobacterium bovis* (BCG) ATCC 27289, *Mycobacterium tuberculosis* (H37) ATCC 27294 and d) Yeast: *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisae* (ATCC 2601). The strains were maintained at 4°C in BHI Agar. Before testing, they were inoculated in BHI agar and incubated for 24 h at 37°C.

Evaluation of antimicrobial activity of the extracts

The antimicrobial activity was evaluated by agar diffusion according to the methodology proposed in document M7-A6 (CLSI, 2003) for bacteria, M24-A2 (CLSI, 2008b) for mycobacteria and M44-A2 (CLSI, 2008a, 2009) for fungi.

For bacteria, Mueller Hinton agar and for yeasts Mueller Hinton agar with 2% glucose were used. The lyophilized extracts were dissolved in DMSO at a final concentration of 50 mg / mL and placed in wells punched into the agar in a volume of 40 microliters.

Then, the medium was inoculated with microorganism's suspensions in saline solution with turbidity corresponding to the 0.5 tube on MacFarland scale. The plates were incubated at 37°C for 24 h.

The activity against *Mycobacterium* was determined by diffusion in agar Middlebrook 7H10 medium added to Middlebrook OADC Enrichment®. The plant extracts at 50 mg/mL concentration, in a volume of 10 µL, was placed on disks of filter paper syrup type at 10 mm diameter and dried at 37°C. The agar Middlebrook 7H10 medium was inoculated with a suspension of *Mycobacterium* with turbidity corresponding to the 2.0 tube of MacFarland scale. The cultures were incubated at 37°C for 28 days.

The minimum inhibitory concentration (MIC) was taken for fungi and bacteria by broth microdilution according to the methodology proposed in document M27-A3. The minimum microbicidal concentration (MMC) was performed using nutrient agar (CLSI, 2008). All experiments were performed in triplicates.

Antioxidant activity of extracts

To determine the antioxidant activity of different concentrations of the extracts (from 400 to 1.56 µg/mL, in serial dilution ratio 2) in an ethanolic solution (2 mL), we admixed 0.5 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (0.5 mM, diluted in ethanol). After incubation for 30 min in the dark and at room temperature, the absorbance was measured at 517 nm. The blank consisted of all reagents except for the extracts. Ascorbic acid, BHT and quercetin were used as a positive control. The scavenging property was calculated as a percentage of DPPH scavenged radicals, using the following equation: scavenging DPPH radical (%) = [(absorbance of blank - absorbance of sample)/ (absorbance blank)] x 100, and also led the

Table 1. Content of total phenolics and flavonoids in the extracts of *F. pumila.*

Extract	Phenolic compounds * (mg GA/g) **	Flavonoids compounds * (mg quercetin/g) ***
Fresh leaf	153.21 ± 8.4^{b}	9.38 ± 0.1^{b}
Dry leaf	154.29 ± 3.5^{b}	15.30 ± 0.6^{a}
Fresh stem	84.8 ± 3.3^{a}	2.56 ± 0.2^{d}
Dry stem	492.57 ± 7.9^{f}	1.29 ± 0.1^{e}
Fresh root	370.24 ± 11.7^{d}	2.71 ± 0.1^{d}
Dry root	724.39 ± 27.7^{g}	1.63 ± 0.1^{e}
Fresh fruit	298.08 ± 6.6^{c}	5.70 ± 0.4^{c}
Dry fruit	407.26 ± 19.1^{e}	3.05 ± 0.1^{d}

*Results expressed as mean ± standard deviation (n = 3). Means with different letters are statistically different in the same column or compound (Scott -Knott p < 0.05). ** Milligrams of gallic acid (GA) per gram of sample. *** Milligrams of quercetin per gram of sample.

EC_{50} of each extract. Values are presented as an average of triplicate independent experiments (Yen et al., 2005).

Evaluation of the cytotoxic activity of extracts on cell culture

Cytotoxicity was assessed by 3 - (4,5-dimethylthiazol-2YL) -2,5-diphenyltetrazolium bromide (MTT) method. In this test, 1 x 10^4 BHK-21 cells (newborn hamster's kidney) per well were seeded in 96-well plates containing the medium Eagle's Minimum Essential (MEM) (plus 10% fetal bovine serum and antibiotics). 0.1 mL of MEM containing 1% fetal bovine serum with decreasing dilutions of the extracts (5 to 0.039 mg/mL) was added to the cultures for 24 h. After incubation, 10 µL of MTT at a concentration of 5 mg/mL was added and incubated for 4 h at room temperature for the MTT incorporation and for the formation of formazan crystals. Spectrophotometric analysis was performed on a microplate reader at 570 nm. The percentage of cytotoxicity was calculated by using the formula [(AB)/ AX100], where A and B are values of the optical densities of the treated and controlled cells, respectively (Araújo et al., 2008). All experiments were performed in triplicates.

Statistical evaluation

The statistical analysis was performed using the SISVAR 5.3 software. Analysis of variance (ANOVA) and also the Scott-Knott test was applied to observe significant differences between average values ($p < 0.05$).

RESULTS

Concerning the phytochemical screening, all extracts showed the presence of tannins and flavonoids, but no anthraquinones, alkaloids or saponins. The highest content of total phenolic compounds was presented by the dried root (724.39 mg GA/g) and the lowest on the fresh stem (84.8 mg GA/g). The highest content of flavonoids was observed on the dried leaf (15.30 mg quercetin/g) and the lowest in the dried stem (1.29 mg quercetin/g) (Table 1).

For antimicrobial activity, it was possible to observe that only *B. subtilis*, *B. cereus*, *M. luteus*, *E. faecalis*, *S. aureus* and *P. mirabilis* were sensitive to the extracts of *F. pumila*. *M. luteus* was the most sensitive bacteria except for the dried leaf extract (halos from 10-16 mm). *B. cereus* was sensitive to all extracts, but showed smaller halos from 6-14 mm. No inhibition was observed for *E. aerogenes*, *E. coli*, *P. aeruginosa*, *S. typhimurium*, *S. marcescens*, *M. bovis*, *M. tuberculosis*, *C. albicans* and *S. cerevisiae*. The best antimicrobial activity was exhibited on the extract of dried root (Table 2).

In assessing the minimum inhibitory concentration (MIC), all microorganisms tested were sensitive to the extracts of *F. pumila*. The results show minimal difference between the MIC prepared with the dried leaves and cooked with fresh plant extracts. The best result for the MIC was for the extract of fresh stem (Table 3).

Regarding the evaluation of minimal microbicidal concentration (MMC) test, the extracts prepared with dried plant exhibited better results, and the most efficient was the dried stem extract (Table 4).

For the DPPH free radical scavenging activity, the highest antioxidant potential was shown on the extract of dried stem, which had the lowest concentration capable of sequestering 50% of DPPH radicals, with EC_{50} of 12.81 µg/mL (Table 5). Correlation between phenolic compounds and antioxidant activity was positive (r^2= 0.32), varying from weak to moderate.

Only the fresh leaf extract showed cytotoxic activity with CC_{50} at 5 mg/mL.

DISCUSSION

Ficus species generally exhibit flavonoids and tannins in their chemical composition (Sirisha et al., 2010). Several studies have reported the presence of flavonoids in extracts of leaf and stem of *F. pumila*, and the presence of tannins in the methanol extract of the leaves (Pistelli et al., 2000; Leong et al., 2008; Kaur, 2012). Thus, the results obtained in this study confirm data previously

Table 2. Antimicrobial activity of extracts of *F. pumila*. Agar diffusion test. Inhibition zones in mm.

Microorganism	Extract							
	Fresh leaf	Dry leaf	Fresh stem	Dry stem	Fresh root	Dry root	Fresh fruit	Dry fruit
Bacillus subtilis	11	10	0	9	0	10	0	0
Bacillus cereus	6	9	11	12	11	14	7	9
Micrococcus luteus	0	11	14	15	14	16	10	11
Enterococcus faecalis	8	9	8	9	9	10	0	9
Staphylococcus aureus	0	7	11	12	11	12	7	13
Escherichia coli	0	0	0	0	0	0	0	0
Enterobacter aerogenes	0	0	0	0	0	0	0	0
Serratia marcescens	0	0	0	0	0	0	0	0
Pseudomonas aeruginosa	0	0	0	0	0	0	0	0
Proteus mirabilis	0	8	10	11	10	11	7	9
Salmonella typhimurium	0	0	0	0	0	0	0	0
Candida albicans	0	0	0	0	0	0	0	0
Saccharomyces cerevisae	0	0	0	0	0	0	0	0
Mycobacterium tuberculosis	0	0	0	0	0	0	0	0
Mycobacterium bovis	0	0	0	0	0	0	0	0

Table 3. Minimal inhibitory concentration (mg/mL) extracts of *F. pumila*.

Microorganism	Extract							
	Fresh leaf	Dry leaf	Fresh steam	Dry steam	Fresh root	Dry root	Fresh fruit	Dry fruit
Bacillus subtilis	3.12	12.5	6.25	6.25	12.5	6.25	6.25	12.5
Bacillus cereus	6.25	6.25	0.39	0.39	0.78	0.78	0.78	0.78
Micrococcus luteus	6.25	6.25	3.12	6.25	0.78	6.25	0.39	3.12
Enterococcus faecalis	6.25	3.12	6.25	3.12	12.5	6.25	6.25	12.5
Staphylococcus aureus	6.25	12.5	6.25	12.5	12.5	12.5	12.5	12.5
Escherichia coli	6.25	3.12	3.12	6.25	12.5	6.25	12.5	6.25
Enterobacter aerogenes	6.25	6.25	3.12	6.25	6.25	6.25	6.25	12.5
Serratia marcescens	6.25	6.25	6.25	3.12	6.25	6.25	6.25	6.25
Pseudomonas aeruginosa	6.25	6.25	6.25	6.25	6.25	6.25	12.5	6.25
Proteus mirabilis	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25
Salmonella typhimurium	6.25	6.25	6.25	6.25	6.25	12.5	6.25	12.5
Candida albicans	3.12	3.12	3.12	6.25	3.12	6.25	3.12	6.25
Saccharomyces cerevisae	6.25	6.25	12.5	6.25	6.25	6.25	6.25	12.5

reported in the literature on the chemical composition of *F. pumila* (Table 1). Regarding the content of flavonoids and phenolic compounds, it is believed that phenolic compounds such as tannins and flavonoids from plants may be related to antioxidant and antimicrobial activities (Einbond et al., 2004; Banerjee et al., 2005; Choi et al., 2006).

In the agar diffusion test conducted to evaluate the antimicrobial activity of the extracts, the dried root extract showed better results for the spectrum of action and inhibition zone diameter. However, in determining the MIC and the MMC, the stem extract was more effective. Furthermore, the *E. coli*, *E. aerogenes*, *S. marcescens*, *P. aeruginosa* and *S. typhimurium* bacteria, as well as the yeast *C. albicans* and *S. cerevisiae* showed no inhibition zone in the agar diffusion test, but were sensitive to extracts of *F. pumila* during the broth microdilution test (MIC) (Table 2). This difference in results may possibly be related to the physical characteristics of the culture method, to the solubility of the compounds of each process and the sensitivity of the method.

In the literature, there are few reports on the antimicrobial activity of *F. pumila*. However, several studies have confirmed the antimicrobial activity of different species of the *Ficus* genus. Studies have described the activity of the methanol extract of fresh

Table 4. Minimal microbicidal concentration (mg/mL) of the extracts of *F. pumila*.

Microorganism	Extract							
	Fresh leaf	Dry leaf	Fresh steam	Dry steam	Fresh root	Dry root	Fresh fruit	Dry fruit
Bacillus subtilis	ND	12.5	ND	12.5	ND	ND	ND	ND
Bacillus cereus	ND	ND	6.25	ND	ND	ND	ND	25
Micrococcus luteus	25	25	12.5	25	ND	12.5	25	25
Enterococcus faecalis	25	12.5	25	12.5	25	12.5	25	12.5
Staphylococcus aureus	12.5	12.5	6.25	12.5	ND	12.5	12.5	12.5
Escherichia coli	25	25	25	12.5	ND	12.5	25	12.5
Enterobacter aerogenes	ND	12.5	ND	6.25	12.5	12.5	12.5	12.5
Serratia marcescens	12.5	ND	ND	25	ND	25	25	25
Pseudomonas aeruginosa	25	25	25	25	ND	25	25	25
Proteus mirabilis	25	12.5	25	12.5	ND	12.5	ND	12.5
Salmonella typhimurium	ND	ND	25	ND	ND	25	25	25
Candida albicans	6.25	6.25	12.5	12.5	6.25	12.5	6.25	6.25
Saccharomyces cerevisae	25	12.5	25	12.5	25	12.5	25	12.5

ND, Not detected at the concentrations used in the test.

Table 5. Antioxidant activity of extracts of *Ficus pumila*.

Extract	% DPPH radicals scavengeing (100 μg/mL)*	EC_{50} (μg/mL)
Fresh leaf	61.85 ± 1.1^a	58.56 ± 0.5^i
Dry leaf	83.18 ± 0.6^e	25.28 ± 0.1^e
Fresh stem	82.93 ± 0.4^e	12.81 ± 0.2^c
Dry stem	81.85 ± 0.4^d	25.79 ± 0.3^f
Fresh root	83.73 ± 0.4^e	25.03 ± 0.1^d
Dry root	81.73 ± 0.5^d	13.20 ± 0.2^c
Fresh fruit	77.13 ± 0.4^c	36.60 ± 0.3^h
Dry fruit	87.59 ± 0.7^f	28.50 ± 0.2^g
Quercetin	81.44 ± 0.2^d	4.57 ± 0.1^a
Ascorbic acid	90.43 ± 0.3^g	6.49 ± 0.1^b
BHT**	63.70 ± 0.1^b	70.11 ± 0.5^j

*Results expressed as mean ± standard deviation (n = 3). Means with different letters are statistically different (Scott -Knott $p < 0.05$).
**BHT: Butylated hydroxytoluene.

leaves of *F. pumila* against *B. subtilis* and methanolic extracts of fruit and stem of *Ficus microcarpa* against Gram-positive and Gram-negative bacteria (Ragasa et al., 1999; Sirisha et al., 2010). The activities of the methanol bark extract of *Ficus glomerata* against *B. subtilis* and ethanol root extracts of *Ficu sracemosa* and *Ficus benghalensis* against *S. aureus* and of the ethanolic root extract of *Ficus racemosa* against *E. coli*, *E. cloacae*, *P. aeruginosa*, *B. subtilis* and *C. albicans* has been mentioned (Murti and Kumar, 2011; Goyal, 2012; Jagtap et al., 2012) .

The antimicrobial activity of different speciesfrom *Ficus sp.*, can be related to the presence of tannins and flavonoids. It is believed that flavonoids are capable of complexing with the bacterial cell wall, causing the death of the microorganism and the tannins are able to inactivate enzymes, transport proteins and microbial adherence (Goyal, 2012).

The antioxidant activity of ethanol extracts of leaf, stem, fruit and root of *F. pumila* was determined by the DPPH method. This technique allows the evaluationof antioxidant activity through the ability to scavenge free radicals in a given period of time. In this case, the antioxidant activity is determined by 50% reduction on the initial concentration of DPPH, or the EC_{50} value. It is considered that the lower the EC_{50} value, the higher the antioxidant activity of the extract. Thus, according to the EC_{50} values, the highest antioxidant activity was exhibited by the extract of fresh stem (Table 3).

The antioxidant activity of plants of the genus *Ficus* is

due to the presence of flavonoids and phenolic compounds in its composition. Studies reported the antioxidant activity of methanol extracts of the stem, fruit and leaves of *F. microcarpa* and aqueous extract of dried stem of *F. glomerata*(Sirisha et al., 2010). A high antioxidant activity of hydroethanolic leaf extract of *F. pumila* was also described which occurred in the presence of phenolic compounds and flavonoids (Leong et al., 2008).

Ficus species are rich in flavonoids and phenolic compounds that produce a strong antioxidant property of these plants, aiding in the prevention and treatment of many diseases (Sirisha et al., 2010). Phenolic compounds act as radical scavengers and sometimes as metal chelators, being probably responsible for the antioxidant activity of *F. pumila* (Moreno and Neuza, 2010).

The positive correlation between the phenolic content and the antioxidant activity indicates that phenolic compounds have an important role in the antioxidant activity presented by the ethanolic extracts of *F. pumila*.

The cytotoxicity evaluation allows determination of the concentration of drug to be used and provides important information about the possible cell damage (Araújo et al., 2008). In this study, the cytotoxic activity of the extracts of *F. pumila* was evaluated in BHK 21 cells. These mammalian cells are derived from baby hamster kidney and were used to determine the cytotoxic activity to various biological compounds (Carbonell et al. 2003; Joshi and Chauhan, 2013). According to the results, only the fresh leaf extracts were cytotoxic for the culture of BHK-21 cells at 5 mg/mL concentration. This cytotoxicity may be related to the high content of flavonoids found in the leaves of *F. pumila* (Xie et al 2009).

There are few studies on the cytotoxicity of *F. pumila*. Ramcharani et al. (2010) have shown cytotoxic activity of methanol leaf and stem extracts of *F. pumila* to the human leukemia cells (MT-4). This activity was attributed to the presence of phenolic compounds. It is believed that these compounds may trigger intracellular signaling pathways that induce death of leukemic cells. The difference in result may be related to the cell type used in the evaluation, since the same methodology was used to assess cell viability.

The results of this study show for the first time that the hydroethanolic extracts of *F. pumila* have active compounds with antioxidant and antimicrobial activities and should be taken into consideration for the development of new drugs.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Microbiological and physicochemical assessments of groundwater quality at Punjab, Pakistan

Ammara Hassan[1]* and M. Nawaz[2]

[1]Pakistan Council of Scientific and Industrial Research, Lahore, Pakistan.
[2]College of Earth and Environmental Sciences, University of the Punjab, Lahore, Pakistan.

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The assessment of groundwater is essential for the estimation of suitability of water for safe use. An attempt has been made to study the groundwater at the district level of Punjab, Pakistan. These samples were analyzed for various water quality parameters like pH, color, odor, conductance, total suspended solids, trace metals (Fe, cu, B, Ba, Al, Cr, Cd, Ni, Mn and Se), ionic concentration (HCO_3, CO_3, Cl, SO_4, Na, K, Ca, Mg, NO_3, NO_2, NH_4, F, PO_4 and CN) and for microbiological enumeration (total viable count, total and fecal coliforms *Escherichia coli, Salmonella* spp. and *Pseudomonas* spp.). The data was analyzed with WHO guidelines/ recommendations. The results of physical analysis indicated that all samples are safe except the groundwater of Kasur and Khanewal District. About 66.67% samples are out of total suspended solids (TSS) limit. Microbiologically, only six groundwater of Punjab districts are found potable according to WHO limits. In the trace metals analysis, highest level of iron was detected in Jhang while the groundwater of three districts were not potable due to high level of boron and nickel but the groundwater of all districts was found safe with respect to Ba, Al and Cr. As far as ionic concentration is concerned, 11 districts were found not to be within WHO limit in the case of bicarbonates, seven in phosphates, five in floride, and eight in calcium and potassium. Only 10 districts fall within the limit of chlorine and all samples showed satisfactory results in the case of CN, CO_3, NO_2, NO_3, Mg, SO_4 and NH_4.

Key words: Groundwater, physicochemical, ionic concentration, traces elements, microbiology, WHO guidelines/recommendations.

INTRODUCTION

The quality of groundwater is the resultant of all the processes and reactions that act on the water from the surroundings and it varies from place to place and with depth of the water table (Reid et al., 2003). Groundwater has many unique characteristics which makes it suitable for public use. It is naturally found in sterile conditions (Mondal et al., 2007). Groundwater particularly accounts for the supply of safe drinking water in rural areas, where

*Corresponding author. E-mail: ahassan.pcsir@gmail.com.

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population is dispersed. For proper infrastructure, a high cost treatment setup and transportation of surface water are needed (Langeneggar, 1990).

Unfortunately, in Pakistan, the availability of groundwater is not unlimited nor is it safe for drinking purpose. The problem of groundwater pollution in several parts of the country has become so acute that extensive groundwater resources may be damaged (Gunkel et al, 2007).

There are many reasons and sources of contamination to the groundwater, for example, land disposal of solid wastes, sewage disposal on land, agricultural activities, urban runoff and polluted surface water (Laluraj et al., 2005). The suitability of groundwater has been examined with reference to WHO (2004) and NEQS (2002) standards.

The quality of groundwater in some parts of the Pakistan is changing as a result of human activities. Bacteria occasionally find their way into groundwater, sometimes in dangerously high concentrations. But freedom from bacteria alone does not mean that the water is fit to drink (Khalafalla, 1993; Babiker and Muhammad, 2014). Many unseen dissolved minerals and organic constituents are present in groundwater in various concentrations. Most are harmless or even beneficial; though occurring infrequently, others are harmful, and few may be highly toxic (Longe and Balogun, 2010). Water typically is not considered fit for drinking if the quality of dissolved minerals exceeds 1000 mg/L. Water with a few thousand mg/L of dissolved minerals is classed as slightly saline, but sometimes used in areas where less-mineralized water is not available (WHO, 2004). These dissolved minerals can be hazardous to animals and plants in large concentration; for example too much sodium in the water may be harmful to the people who have heart problem. Boron is the mineral that is good for plants in small amount but it is toxic to some plants in only slight higher concentration. Water that contains a lot of calcium and magnesium is said to be hard. The very hard water is not desirable for many domestic uses; it will leave a scaly deposit on the inside of pipes, boilers and tanks. Groundwater, especially if acidic, in many places contains excessive amount of iron. Iron causes reddish stain on plumbing fixtures and clothing (Mondal et al., 2007).

In recent years, the growth of industry, technology, population and water use has increased the stress on both our land and water resources. Locally, the quality of groundwater has been degraded (Langeneggar, 1990). Municipal and industrial wastes and chemical fertilizers, herbicides and pesticides not properly curtailed have entered into the soil, infiltered some aquifers, and degraded the groundwater quality. Other pollution problems include sewage leakage, faulty septic-tank operation and landfill leachates (Woods, 1990).

In this study, the portability of groundwater of 36 districts of the Punjab province, Pakistan has been assessed for physical, chemical and microbiological quality.

MATERIALS AND METHODS

Samples collection

The samples were collected during March-September, 2012 from different locations of 36 districts of Punjab that are being extensively used for drinking and other domestic purposes (Figure 1). The water samples were taken from average depth of 25-35 m. All water samples were collected in sterile glass Schott bottles (5 L). While sampling, some parameters like pH and conductance were measured in the field by using portable kits. For other parameters, samples were stored and transported in a cool box kept below 4°C (APHA, 2005). The detailed chemical and microbiological analyses were performed as soon as the samples were carried to the laboratory. All tests were performed in triplicate on the random appropriate sampling from each district.

Microbiological analysis

All samples were examined using standard methods of APHA (2005).

Physicochemical analysis

The physico-chemical parameters were determined according to procedures outlined in the Standard Methods for the Examination of Water and Wastewater (APHA, 2005). Turbidity was measured with a HACH 2100 P Turbidimeter. Sodium and potassium were measured by flame emission photometry, trace metals by atomic absorption spectrophotometer, calcium and magnesium by EDTA titration, sulphate by the turbidimetric method, colour by colour comparator and chloride by argentometric tittration. Other analyses included alkalinity by strong acid titration method. Nutrients (nitrate-nitrogen and phosphate-phosphorous) were determined using Dionex-80 ion analyzer and ammonium by direct nesslerisation and spectrophoto-metric determination at 410 nm. Fluoride was determined by SPADNS method, total dissolved solids and suspended solids were measured gravimetrically after drying in an oven to a constant weight at 105°C. Table 1 shows the name of districts of the Punjab, from where the samples of water were collected, with their area and population density (people/km^2).

RESULTS AND DISCUSSION

Physical analysis

As far as physical parameters of the collected samples are concerned, the color and odor of all samples are within the WHO limits (WHO, 2004) except the groundwater of Kasur District. The pH dropped to 5.20 in groundwater of Kasur while the samples collected from Khanewal was found to be alkaline with pH of 8.83 while the rest samples fall within the recommended range (6.5-8.5). According to WHO guidelines, the total suspended solids (TSS) should be zero; but about 66.67% samples are out of range. In this study, the conductivity of the collected samples is observed above permissible limits of WHO. The results of physical parameters of selected

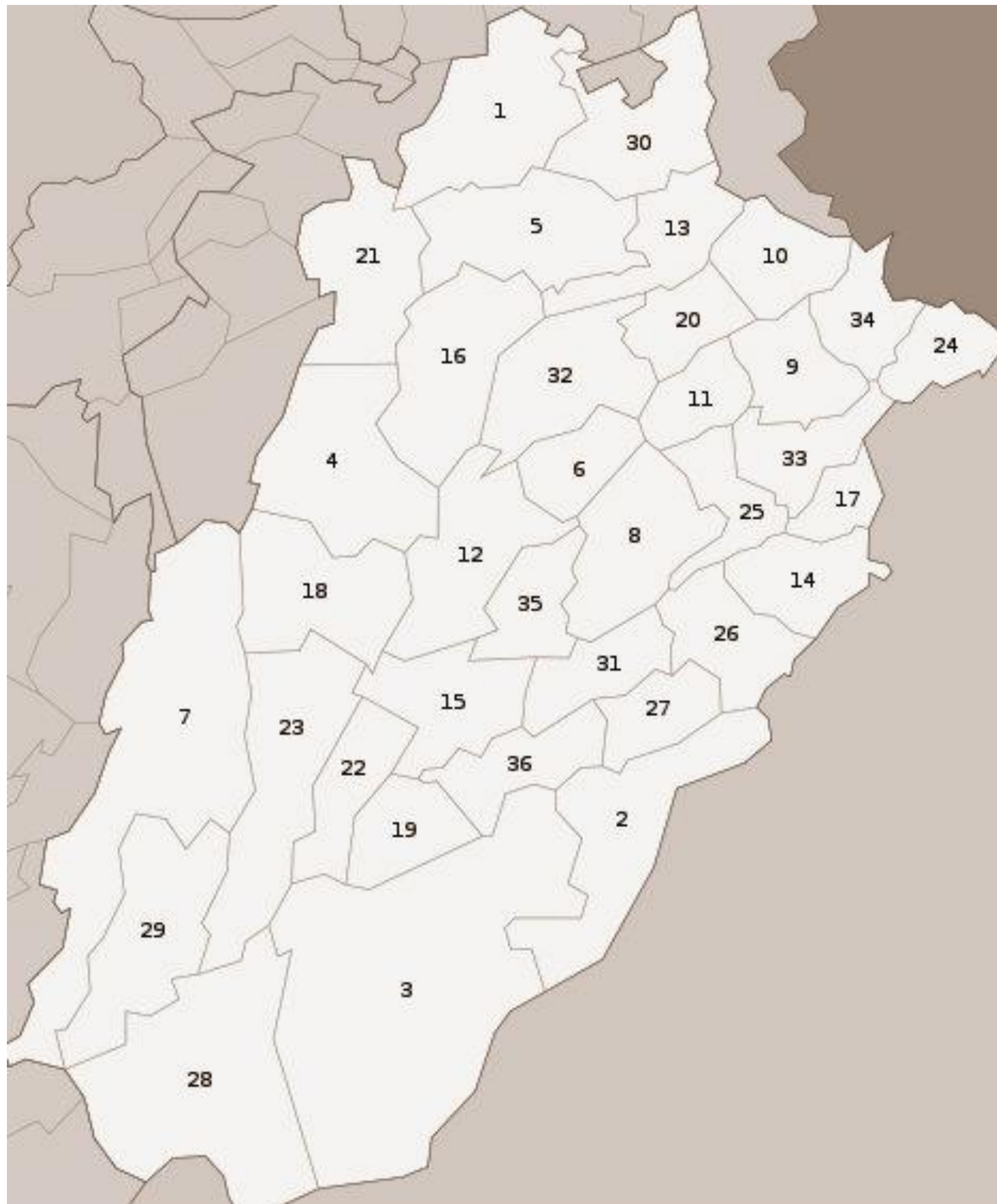

Figure 1. Map of districts of Punjab (refer to Table 1).

samples, along with WHO recommended limits are shown in Table 2.

Microbiological analysis

The most basic test for bacterial contamination of a water supply is the test for total coliform bacteria (Styenberg et al., 1995). Total coliform counts give a general indication of the sanitary condition of a water supply. Total coliforms include bacteria that are found in the soil and in water that has been influenced by surface water, and in human or animal waste (Atherholt et al., 2003). Fecal coliforms are the group of the total coliforms that are considered to be present specifically in the gut and feces of warm-blooded animals (Crysup and Mott, 2001). If fecal coliform counts are high (over 200 colonies per 100 ml of water sample), there is a greater chance that pathogenic organisms are also present. Diseases and illnesses such as typhoid fever, hepatitis, gastroenteritis, dysentery and ear infections can be contracted in waters with high fecal coliform counts (Francy et al., 2000). According to WHO recommendations/ guidelines, drinking water must have zero total coliforms, fecal coliforms and *E. coli* (WHO, 2004). The results of the present study show that only 22% groundwater samples are found according to the

Table 1. Information on the selected districts of Punjab.

District name	Area (km^2)	Population (1998)	Density (people/km^2)
Attock	6,858	1,274,935	186
Bahawalnagar	8,878	2,061,447	232
Bahawalpur	24,830	2,433,091	98
Bhakkar	8,153	1,051,456	129
Chakwal	6,524	1,083,725	166
Chiniot		965,124	
Dera Ghazi Khan	11,922	1,643,118	138
Faisalabad	5,856	5,429,547	927
Gujranwala	3,622	3,400,940	939
Gujrat	3,192	2,048,008	642
Hafizabad	2,367	832,980	352
Jhang	8,809	2,834,545	322
Jhelum	3,587	936,957	261
Kasur	3,995	2,375,875	595
Khanewal	4,349	2,068,490	476
Khushab	6,511	905,711	139
Lahore	1,772	6,318,745	3,566
Layyah	6,291	1,120,951	178
Lodhran	2,778	1,171,800	422
Mandi Bahauddin	2,673	1,160,552	434
Mianwali	5,840	1,056,620	181
Multan	3,720	3,116,851	838
Muzaffargarh	8,249	2,635,903	320
Narowal	2,337	1,265,097	541
Nankana Sahib	2,960	1,410,000	
Okara	4,377	2,232,992	510
Pakpattan	2,724	1,286,680	472
Rahim Yar Khan	11,880	3,141,053	264
Rajanpur	12,319	1,103,618	90
Rawalpindi	5,286	3,363,911	636
Sahiwal	3,201	1,843,194	576
Sargodha	5,854	2,665,979	455
Sheikhupura	5,960	3,321,029	557
Sialkot	3,016	2,723,481	903
Toba Tek Singh	3,252	1,621,593	499
Vehari	4,364	2,090,416	479

limit of WHO for total coliforms, only eight districts are found free of *E. coli*. The 13 districts are found contaminated with *Salmonella* spp. while only eight districts groundwater was free from the *Pseudomonas* spp. Overall, only six groundwater of Punjab districts are found potable according to WHO limits. The results of microbiological analysis are shown in Table 3.

Trace metals analysis

The results of trace metals are shown in Table 4, according to this study, the highest level of iron (Fe) was detected in Jhang (0.89) and overall groundwater of 17 districts were found to be outside the range of recommended limits of WHO. Infact, iron is one of the most worrying elements in water supplies (Pradhan et al., 2001). Rainwater as it infiltrates the soil and underlying geologic formations dissolves iron, causing it to seep into aquifers that serve as sources of groundwater for wells (Marian, 1991). Iron and manganese (Mn) are common water contaminants that are not considered health hazards. Their presence in water results in staining as well as offensive tastes and appearances. As groundwater flows through sediments, metals such as iron and manganese (Mn) are dissolved and may later be found

Table 2. Physical parameters for 36 districts of Punjab.

Location	Color	Odor	pH at 26°C	Conductivity (µs/cm)	TSS (mg/L)
Attock	Colorless	Odorless	8.23	679	N.D
Bahawalnagar	Colorless	Odorless	8.29	685	N.D
Bahawalpur	Colorless	Odorless	8.03	46300	36.0
Bhakkar	Colorless	Odorless	7.81	887	N.D
Chakwal	Colorless	Odorless	8.19	276	N.D
Chiniot	Colorless	Odorless	8.44	12960	52.0
Dera Ghazi Khan	Colorless	Odorless	7.20	140	N.D
Faisalabad	Colorless	Odorless	8.23	889	N.D
Gujranwala	Colorless	Odorless	8.29	112	N.D
Gujrat	Colorless	Odorless	8.03	5566	61.0
Hafizabad	Colorless	Odorless	7.81	678	N.D
Jhang	Colorless	Odorless	8.19	431	N.D
Jhelum	Colorless	Odorless	8.44	569	N.D
Kasur	Colored	Odorless	5.20	9888	43.9
Khanewal	Colorless	Odorless	8.83	8886	N.D
Khushab	Colorless	Odorless	8.09	909	N.D
Lahore	Colorless	Odorless	8.04	213	23.6
Layyah	Colorless	Odorless	7.25	2006	19.9
Lodhran	Colorless	Odorless	8.11	334	N.D
Mandi Bahauddin	Colorless	Odorless	8.49	554	55.1
Mianwali	Colorless	Odorless	7.29	665	N.D
Multan	Colorless	Odorless	7.20	881	N.D
Muzaffargarh	Colorless	Odorless	8.03	332	N.D
Narowal	Colorless	Odorless	8.01	321	39.0
Nankana Sahib	Colorless	Odorless	7.24	456	N.D
Okara	Colorless	Odorless	8.29	667	N.D
Pakpattan	Colorless	Odorless	7.44	119	N.D
Rahim Yar Khan	Colorless	Odorless	7.20	443	N.D
Rajanpur	Colorless	Odorless	7.23	663	N.D
Rawalpindi	Colorless	Odorless	8.21	459	22.9
Sahiwal	Colorless	Odorless	8.00	442	16.0
Sargodha	Colorless	Odorless	7.89	112	N.D
Sheikhupura	Colorless	Odorless	8.09	11001	N.D
Sialkot	Colorless	Odorless	7.44	445	22.0
Toba Tek Singh	Colorless	Odorless	7.01	111	N.D
Vehari	Colorless	Odorless	6.99	236	45.9
WHO	Colorless	Odorless	6.5-8.5	14.00	N.D

in high concentrations in water. Industrial discharges, urban activities, agriculture, groundwater pumages and disposal of waste can all affect groundwater quality (Kjoller et al., 2004).

Copper is a metal that occurs naturally in rock, soil, plants, animals and water (Kerbyson and Schandorf, 1966). The level of copper in surface and groundwater is generally very low. High levels of copper may get into the environment through mining, farming, manufacturing operations and municipal or industrial wastewater releases into rivers and lakes. Copper can get into drinking water either directly by contaminating well water or through corrosion of copper pipes if water is acidic. Corrosion of pipes is by far the greatest cause for concern. In human, its causes stomach and intestinal diseases, liver and kidney damage in high doses. But in this study, fortunately all samples were found within limits of WHO for copper concentration.

Boron is the mineral that is good for plants in small amount but it is toxic to some plants in only slight higher concentration and nickel may be found in slate, sandstone, clay minerals and basalt. The main nickel source is pentlandite (Atherholt et al., 2003). Nickel is a dietary requirement for a number of organisms, therefore

Table 3. Microbiological analysis of groundwater samples.

Location	Viable count/ml	Total coliforms (MPN/100 ml)	Fecal coliforms (MPN/100 ml)	*E. coli* (MPN/100 ml)	*Salmonella* spp. /25 ml	*Pseudomonas* spp. (MPN/100ml)
Attock	$90*10^3$	94	70	33	Detected	17
Bahawalnagar	$8.5*10^2$	24	20	23	N.D	23
Bahawalpur	$1.9*10^4$	94	49	80	Detected	23
Bhakkar	$1.2*10^2$	N.D	N.D	N.D	N.D	N.D
Chakwal	$5.0*10^2$	N.D	N.D	N.D	N.D	N.D
Chiniot	$6.2*10^2$	70	39	31	N.D	17
Dera Ghazi Khan	$23*10^3$	84	70	33	N.D	11
Faisalabad	$4.5*10^2$	24	20	23	N.D	23
Gujranwala	$9.9*10^4$	40	20	20	Detected	4.0
Gujrat	$1.4*10^2$	14	14	N.D	N.D	2.0
Hafizabad	$5.5*10^3$	N.D	N.D	N.D	N.D	N.D
Jhang	$6.7*10^2$	40	17	31	Detected	6.8
Jhelum	$60*10^3$	70	63	33	Detected	11
Kasur	$8.7*10^1$	33	20	23	N.D	23
Khanewal	$3.9*10^4$	94	49	80	Detected	20
Khushab	$1.5*10^2$	N.D	N.D	N.D	N.D	N.D
Lahore	$5.9*10^2$	14	11	4.5	N.D	4.5
Layyah	$1.2*10^2$	21	11	11	Detected	17
Lodhran	$50*10^2$	94	70	33	N.D	14
Mandi Bahauddin	$8.5*10^2$	24	20	23	N.D	23
Mianwali	$1.0*10^4$	94	49	80	Detected	33
Multan	$1.5*10^2$	11	6.8	2.0	N.D	N.D
Muzaffargarh	$5.4*10^5$	N.D	N.D	N.D	N.D	N.D
Narowal	$6.5*10^2$	48	39	31	Detected	17
Nankana Sahib	$76*10^3$	70	47	33	Detected	6.8
Okara	$8.4*10^2$	24	20	23	N.D	23
Pakpattan	$1.1*10^4$	84	49	80	N.D	33
Rahim Yar Khan	$1.3*10^4$	120	110	39	Detected	N.D
Rajanpur	$5.2*10^2$	33	33	N.D	N.D	2.0
Rawalpindi	$2.2*10^2$	26	11	17	N.D	17
Sahiwal	$40*10^3$	70	63	33	N.D	20
Sargodha	$1.5*10^1$	17	20	23	N.D	23
Sheikhupura	$6.9*10^4$	63	49	33	Detected	7.8
Sialkot	$2.2*10^2$	N.D	N.D	N.D	N.D	2.0
Toba Tek Singh	$3.9*10^2$	N.D	N.D	N.D	N.D	N.D
Vehari	$3.1*10^4$	40	11	24	Detected	14
WHO	-	Zero	Zero	Zero	N.D	Zero

*N.D = Not detected.

it might be of significance to humans (MacCutcheon et al., 1983). Nickel compounds may be toxic in high concentrations, but these are often water insoluble, limiting potential harm (Singh and Lawrence, 2007). Results show that the groundwater of three districts could not be potable due to high level of boron (B) and Ni in groundwater.

Four districts are found not to be within WHO limits for cadmium which is found in rocks, coals and petroleum and enters the ground and surface water when dissolved by acidic waters. It causes kidney damage, anemia, high blood pressure and liver damage.

Selenium (Se) occurs naturally in the environment. Selenium that is immobile and will not dissolve in water is less of a risk to organisms. The oxygen levels in the soil and the acidity of the soil will increase mobile forms of selenium. Higher oxygen levels and increased acidity of soils is usually cause by human activities such as industrial and agricultural processes (Kerbyson and Schandorf, 1966). Humans may be exposed to selenium

Table 4. Trace metals (mg/L) analysis of groundwater samples.

Location	Fe	Cu	B	Ba	Al	Cr	Cd	Ni	Mn	Se
Attock	0.78	N.D	0.214	N.D	N.D	N.D	0.01	0.29	0.09	N.D
Bahawalnagar	0.11	N.D	0.4	0.2	N.D	N.D	N.D	N.D	0.02	N.D
Bahawalpur	0.34	N.D	0.47	0.1	N.D	N.D	N.D	N.D	N.D	N.D
Bhakkar	0.36	N.D	1.91	0.1	N.D	N.D	N.D	N.D	N.D	N.D
Chakwal	0.21	0.055	N.D	N.D	N.D	N.D	N.D	0.01	N.D	N.D
Chiniot	0.33	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Dera Ghazi Khan	0.71	N.D	0.20	N.D	N.D	N.D	N.D	0.25	N.D	N.D
Faisalabad	0.35	N.D	0.46	0.2	N.D	N.D	N.D	N.D	N.D	N.D
Gujranwala	0.34	N.D	N.D	0.1	N.D	N.D	N.D	N.D	N.D	N.D
Gujrat	0.22	N.D	N.D	0.1	N.D	N.D	N.D	N.D	N.D	N.D
Hafizabad	0.21	0.051	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Jhang	0.89	N.D	N.D	N.D	N.D	N.D	0.03	N.D	0.01	N.D
Jhelum	0.71	N.D	0.46	N.D	N.D	N.D	N.D	N.D	0.10	N.D
Kasur	0.15	N.D	0.55	0.2	N.D	N.D	N.D	N.D	N.D	N.D
Khanewal	0.25	N.D	0.42	0.1	N.D	N.D	N.D	0.14	N.D	N.D
Khushab	0.16	N.D	0.33	0.1	N.D	N.D	N.D	N.D	N.D	N.D
Lahore	0.26	0.021	0.41	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Layyah	0.30	N.D	N.D	N.D	N.D	N.D	N.D	N.D	0.03	N.D
Lodhran	0.77	N.D	0.14	N.D	N.D	N.D	0.02	N.D	N.D	N.D
Mandi Bahauddin	0.19	N.D	N.D	0.2	N.D	N.D	N.D	N.D	N.D	N.D
Mianwali	0.36	N.D	N.D	0.1	N.D	N.D	N.D	N.D	N.D	N.D
Multan	0.16	0.022	1.99	0.1	N.D	N.D	N.D	N.D	N.D	N.D
Muzaffargarh	0.26	0.020	0.4	N.D	N.D	N.D	N.D	0.01	N.D	N.D
Narowal	0.34	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Nankana Sahib	0.28	N.D	0.11	N.D	N.D	N.D	N.D	0.21	N.D	N.D
Okara	0.10	N.D	0.41	0.2	N.D	N.D	N.D	N.D	0.02	N.D
Pakpattan	0.35	N.D	0.42	0.1	N.D	N.D	N.D	N.D	N.D	N.D
Rahim Yar Khan	0.46	N.D	1.12	0.1	N.D	N.D	N.D	N.D	N.D	N.D
Rajanpur	0.25	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Rawalpindi	0.32	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D	N.D
Sahiwal	0.74	N.D	0.81	N.D	N.D	N.D	0.01	0.03	N.D	N.D
Sargodha	0.11	N.D	0.41	0.2	N.D	N.D	N.D	N.D	N.D	N.D
Sheikhupura	0.45	N.D	0.70	0.1	N.D	N.D	N.D	N.D	N.D	N.D
Sialkot	0.26	N.D	1.60	0.1	N.D	N.D	N.D	N.D	N.D	N.D
Toba Tek Singh	0.21	0.028	0.10	N.D	N.D	N.D	N.D	0.02	N.D	N.D
Vehari	0.31	N.D	N.D	N.D	N.D	N.D	N.D	N.D	0.02	N.D
WHO	0.30	1.0	1.0	1.0	0.02	0.10	0.00	0.05	0.10	N.D

in several ways. Fortunately, all districts groundwater were found safe in the sense of Se.

Barium can cause a variety of cardiac, gastrointestinal and neuromuscular effects. Aluminum occurs naturally in some rocks and drainage from mines. It can precipitate out of water after treatment, causing increased turbidity or dissolved water (Marian, 1991). Chromium has the potential to damage liver, kidney, circulatory and nerve tissues; and cause skin irritation. When released into land, chromium compounds bind to soil and are not likely to migrate to groundwater. They are very persistent in water as sediments. There is a high potential for accumulation of chromium in aquatic life (Singh and Lawrence, 2007) and the results has shown that groundwater of all districts was found safe with respect to Ba, Al, and Cr.

Ions concentration analysis

The presence of carbonates, bicarbonates and hydroxides are the main causes of the alkalinity in the natural water. Bicarbonates represent the major form since they are formed in considerable amount in the soil (MacCutcheon et al., 1983). In this study, we found that 11 districts were outside the WHO limit.

While phosphates limit was exceeded in seven districts, fluoride in five districts, and calcium in eight districts. The fluoride concentration is present in soil strata and the accumulation of fluoride in soil strata results in the leaching process due to percolating water pressure resulting to increased fluoride concentration in groundwater (O'Neil, 1993). When the concentration of phosphates rises, the coagulation processes in drinking water treatment plants may be adversely affected. Manmade sources of phosphate include human sewage, agricultural run-off from crops, sewage from animal feedlots, pulp and paper industry, vegetable and fruit processing, chemical and fertilizer manufacturing and detergents (Marian, 1991).

Only 10 districts samples were found in the limit in the case of chlorine (Cl) and all samples showed satisfactory results in the case of CN, CO_3, NO_2, NO_3, Mg, SO_4 and NH_4. Nitrate is effective plant nutrient and moderately toxic and is considered important for its adverse health effects (Jain and Ram, 1996). The higher nitrate concentration may be attributed to combined effect of contamination from domestic sewage and runoff from fertilized fields (Langanegger, 1987) and cyanides are generally not persistent when released into water or soil, and are not likely to accumulate in aquatic life. They rapidly evaporate and are broken down by microbes. They do not bind to soils and may leach to groundwater. EPA has found cyanide to potentially cause the following health effects when people are exposed to it at levels above the MCL for relatively short periods of time: rapid breathing, tremors and other neurological effects (Marian, 1991).

In the present study, all samples showed satisfactory results for ammonia. NH_3 is the principal form of toxic ammonia. It has been reported toxic to fresh water organisms at concentrations ranging from 0.53 to 22.8 mg/L (DWAF, 1998). Toxic concentrations of ammonia in humans may cause loss of equilibrium, convulsions, coma and death (Bolalak and Frankowaski, 2003). The sulphate contents of water may change significantly with time during infiltration of rainfall and groundwater recharge, which mostly takes place from stagnant pools, puddles and surface runoff water collected in low-lying areas. All samples showed satisfactory results in this study. All samples showed satisfactory results for sodium in this study except that from Gujrat. In fact, sodium concentration more than 50 mg/L make the water unsuitable for domestic use.

Eight districts were found to have unfit groundwater in the case of potassium, with highest value in Mianwali (120 mg/L), which naturally occurs in various minerals, from which it may be dissolved through weathering processes (Chapman, 1996). Potassium plays a central role in plant growth, and it often limits it. Potassium from dead plant and animal material is often bound to clay minerals in soils, before it dissolves in water (O'Neil 1993).

Conclusion

This study has provided information on the water quality status of groundwater from 36 districts of the Punjab, Pakistan. The physical, chemical and microbiological qualities were assessed. The results of physical analysis indicated that pH, color and odor were all within their natural background levels of 6.5-8.5 except the groundwater of Kasur and Khanewal District. According to WHO guidelines, the total suspended solids (TSS) should be zero but about 66.67% samples are not within this limit. In this study, the conductivity of all the collected samples is observed above permissible limits of WHO.

According to WHO recommendations/guidelines, drinking water must have zero total coliforms, fecal coliforms and *E. coli* (WHO, 2004). Results of the present study show that only 22% groundwater samples are found according to the limit of WHO for total coliforms, only eight districts are found free of *E. coli*. 13 districts are found to samples contaminated with *Salmonella* spp., while only eight districts groundwater were free from the *Pseudomonas* spp. Overall, only six groundwater samples of Punjab districts are found potable according to WHO limits.

In the trace metals analysis, highest level of iron (Fe) was detected in Jhang (0.89) and overall groundwater of 17 districts were found to be out of the recommended limits of WHO while the groundwater of three districts could not be potable due to high level of boron and Ni in groundwater. The results of the present study has shown that groundwater of all districts was found safe with respect to Ba, Al and Cr.

As far as ionic concentration is concerned, the 11 districts were found not to be within the WHO limit in the case of bicarbonates while phosphates limit was exceeded in seven districts, fluoride limit was exceeded in five districts, and calcium limit was exceeded in eight districts Table 5. Only ten districts samples were found within the limit in the case of chlorine (Cl) and all samples showed satisfactory results in the case of CN, CO_3, NO_2, NO_3, Mg, SO_4 and NH_4 and eight districts are found to have unfit groundwater in the case of potassium, with highest value in Mianwali (120 mg/L).

Conflict of interest

The authors declare that they have no conflict of interest.

Table 5. Ionic concentration (mg/L) analysis of groundwater samples

Location	HCO_3	CO_3	Cl	SO_4	Na	K	Ca	Mg	NO_3	NO_2	NH_4	F	PO_4	CN
Attock	261.8	N.D	35.0	51.6	116	1.57	8.0	3.4	N.D	N.D	N.D	0.15	N.D	N.D
Bahawalnagar	225.0	N.D	27.2	29.0	95	5.09	14.06	8.54	50.0	N.D	N.D	N.D	N.D	N.D
Bahawalpur	233.6	N.D	121.0	69.9	248	110	820	11.5	N.D	N.D	N.D	N.D	N.D	N.D
Bhakkar	356.0	N.D	80.97	79.0	86	6.0	40	15.5	N.D	N.D	0.85	0.25	0.25	N.D
Chakwal	121.3	N.D	19.9	11.0	10.2	2.1	16	14.58	N.D	N.D	N.D	1.5	N.D	N.D
Chiniot	583.7	N.D	2603	78.0	180	40.8	102	191.0	N.D	N.D	1.02	N.D	N.D	N.D
Dera Ghazi Khan	241.8	N.D	35.9	12.6	121	1.52	8.0	3.1	N.D	N.D	N.D	0.12	N.D	N.D
Faisalabad	229.0	N.D	270.2	29.0	9.51	59.1	14.06	8.4	5.0	N.D	N.D	N.D	N.D	N.D
Gujranwala	222.6	N.D	221.0	67.0	222	90	820	16.5	N.D	N.D	N.D	N.D	1.02	N.D
Gujrat	339.0	N.D	180.9	41.0	281	63.0	40	25.5	N.D	N.D	N.D	N.D	N.D	N.D
Hafizabad	126.0	N.D	19.2	48.0	100	32.0	16	12.21	N.D	N.D	N.D	2.52	N.D	N.D
Jhang	512.5	N.D	280.1	23.0	181	10.0	102	17.9	N.D	N.D	N.D	N.D	N.D	N.D
Jhelum	221.8	N.D	35.1	24.6	102	1.23	8.0	3.1	N.D	N.D	N.D	0.15	N.D	N.D
Kasur	223.0	N.D	22.9	15.0	91	5.01	14.06	8.4	N.D	N.D	N.D	N.D	N.D	N.D
Khanewal	133.6	N.D	121.1	61.0	24.8	112	820	11.5	N.D	N.D	N.D	N.D	N.D	N.D
Khushab	219.0	N.D	820.9	41.0	85.1	60.0	40	15.5	N.D	N.D	0.81	N.D	N.D	N.D
Lahore	131.3	N.D	192.9	22.0	10.21	2.12	16	11.58	N.D	N.D	1.00	2.5	0.50	N.D
Layyah	623.7	N.D	260.1	23.0	124	40.3	102	13.0	N.D	N.D	1.06	4.1	N.D	N.D
Lodhran	281.8	N.D	35.01	65.6	11.3	1.51	8.0	3.6	N.D	N.D	N.D	0.11	N.D	N.D
Mandi Bahauddin	229.0	N.D	27.98	11.0	9.25	59.0	14.06	2.4	5.12	N.D	N.D	N.D	N.D	N.D
Mianwali	231.6	N.D	129.1	61.0	243	120	820	10.5	N.D	N.D	N.D	N.D	N.D	N.D
Multan	322.0	N.D	10.97	89.0	250	6.0	40	11.5	N.D	N.D	N.D	N.D	N.D	N.D
Muzaffargarh	145.3	N.D	101.9	21.0	123	2.0	16	9.50	N.D	N.D	N.D	N.D	0.51	N.D
Narowal	589.7	N.D	269.1	75.0	142	20.9	102	11.0	N.D	N.D	1.02	N.D	N.D	N.D
Nankana Sahib	261.1	N.D	35.1	90.6	216	1.87	8.0	3.49	N.D	N.D	N.D	0.15	N.D	N.D
Okara	221.3	N.D	37.9	22.0	25	5.21	14.06	8.11	5.0	N.D	N.D	0.28	N.D	N.D
Pakpattan	232.8	N.D	421.0	140.0	142	119	820	22.5	1.18	N.D	N.D	N.D	N.D	N.D
Rahim Yar Khan	319.0	N.D	280.1	35.0	231	6.96	40	19.5	N.D	N.D	N.D	N.D	N.D	N.D
Rajanpur	123.1	N.D	49.2	24.0	129	2.33	16	13.5	N.D	N.D	N.D	2.5	N.D	N.D
Rawalpindi	581.7	N.D	260.3	92.0	180	40.3	102	11.0	N.D	N.D	N.D	N.D	N.D	N.D
Sahiwal	261.2	N.D	350.1	21.6	189	1.51	8.0	3.47	N.D	N.D	N.D	0.19	N.D	N.D
Sargodha	222.1	N.D	76.2	20.0	90.4	52.0	14.06	8.14	50.0	N.D	N.D	N.D	N.D	N.D
Sheikhupura	233.3	N.D	12.11	61.0	243	11.0	820	6.5	4.18	N.D	N.D	N.D	1.55	N.D
Sialkot	329.1	N.D	89.8	49.0	120	26.0	40	15.5	N.D	N.D	N.D	1.25	0.25	N.D
T.K Singh	122.3	N.D	11.6	24.0	67.9	20.0	16	14.8	N.D	N.D	N.D	2.1	0.50	N.D
Vehari	583.9	N.D	26.01	96.0	231	40.0	102	1.91	N.D	N.D	1.01	5.0	N.D	N.D
WHO	300	N.D	200-600	250	250	50	250	50	50	50	50	1.5	N.D	N.D

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Full Length Research Paper

Expression of two loopsV3 from HIV-1 fused to cholera toxin A2B subunit using *Lactococcus lactis* as a vector to induce immunity in mucosa

Itza Eloisa Luna-Cruz*, Moises Armides Franco-Molina, Cristina Rodríguez-Padilla, Reyes Tamez-Guerra and Juan Manuel Alcocer-González

Universidad Autónoma de Nuevo León,UANL, Facultad de Ciencias Biológicas Laboratorio de Inmunología y Virología.San Nicolás de los Garza, Ciudad Universitaria, S/N, C.P. 66451. México.

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***Lactococcus lactis*, a nonpathogenic bacterium has been used widely as a vector to deliver antigens to the mucosa; one of the principal threats to world health is AIDS and HIV infection, involving the passage of the virus across a mucosal surface. We used *L. lactis* to express V3 loop from HIV-1, A2B subunit of cholera toxin and their fusion with one or two loops V3 (V3A2B and V3V3A2B). Six-weeks-old Balb/c mice were orally immunized with the recombinant bacteria. Samples of serum, intestinal washes and PBMC lymphocytes were collected to detect IgA and IgG anti-V3, and IL-2 respectively. The recombinants that express the V3 alone or fused to the A2B induced anti-V3 IgA antibodies and anti-V3 IgG antibodies in serum and intestinal washes but recombinant V3V3A2B was the most efficient and the only one that induced the expression of IL-2 in PBMC when compared with the controls ($p<0.05$). We induced anti V3 IgG and IgA and IL-2 in mice immunized with *L. lactis* recombinants that express the antigen V3 fused to A2B subunit of CT; which suggests that this strategy could be used to induce immune response specific of HIV.**

Key words: HIV, vaccines, *Lactococcus lactis*, lactic acid bacteria, mucosal immunity.

INTRODUCTION

Candidate vaccines for HIV can be divided broadly into five categories: synthetic peptides or peptide subunits, live recombinant vectors, live attenuated vaccines, whole inactivated particles and DNA vaccines. Recently, over a hundred vaccine candidates in clinical trials were evaluated, but only three are in clinical phase III. Most vaccines include recombinants vectors, mainly adenovirus, adeno-associated, alphavirus, poxvirus and flavovirus; recombinants bacteria are also used, such as *Salmonella* (IAVI Report, 2014) and *Escherichia coli*, to express gp120 by their easy production and recently results are shown to elicit broad neutralizing sera against HIV-1 in small animals (Bhattacharyya et al., 2013). The use of commensal bacteria as a vector has certain advantages

*Corresponding author. E-mail: itza.lunac@uanl.mx. Fax: (81) 83524212.

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because it has been used in the food industry for years and can be administered orally.

Lactococcus lactis is a noninvasive, nonpathogenic, Gram-positive bacterium that has a long history of use in the production of fermented milk products. *L. lactis* lacks the ability to multiply *in vivo* except in gnotobiotic mice. However, genetically modified *L. lactis* have been effective in delivering antigens to the mucosal immune system and inducing a local immune response. Live *L. lactis* have been shown to pass rapidly through the gastrointestinal tract of animals and humans without colonization (Medina et al., 2008).

Recently, based on its suitable safety profile, *L. lactis* has been used as a vaccine vector to express different types of antigens (Xin et al., 2003; Groot et al., 2008; Yi-jing et al., 2010); this capacity was tested in a phase I study that employed *L. lactis* to express IL-10 in humans (Braat et al., 2006). Gastrointestinal, respiratory and genitourinary mucosal tissues serve as portals of entry for many pathogens, including sexually transmitted pathogens such as HIV. More than 90% of global HIV-1 transmission occurs across a mucosal surface (Griffin et al., 1996). Conserved antibodies epitopes located in the *Env* region on the viral protein gp120 have been identified based on their recognition by neutralizing monoclonal antibodies (mAbs). One of these epitopes is the V3 loop of gp120, contain conserved elements that play a role in virus attachment and which is critical for virus infectivity. Therefore, the induction of antibodies via the V3 loop is important to prevent infection (Hioe et al., 2010). Several strategies have been developed using the V3 loop as an antigen to induce immunity against HIV, in bacterial systems, including *Mycobacterium* (Joseph et al., 2010), *Salmonella* (Chin'ombe et al., 2009) and *Brucella* (Golding et al., 2002). Adjuvants are substances that enhance the immune response to a vaccine. Cholera toxin (CT) is an 86-kDa enterotoxin produced by *Vibrio cholera*, and this toxin is an exceptionally potent mucosal immunogenic (Vajdy and Lycke, 1992). CT can act as an adjuvant to promote the long-term immunological memory to unrelated immunogens in the gut mucosa after oral immunization. CT consists of five B subunits (CTBs), arranged as a pentamer and covalently linked to a single 33-kDa A subunit (CTA) (Kim et al., 1998). The use of CTB fused to the V3 region has been previously demonstrated in a transgenic potato (Kim et al., 2004). To date, few studies concerning the use of *L. lactis* for the presentation of HIV-1 antigens (Xin et al., 2003) have been published. The present study was focused on the strategy to use *L. lactis* to express HIV-1 antigen V3 loops fused to CTB to induce HIV-1 specific response of antibodies and cytokines through oral immunization.

MATERIALS AND METHODS

Bacterial strains and growth media

E. coli DH5 alpha was used as a host strain for plasmid construction and was grown in Luria-Bertani broth (Invitrogen, Carlsbad, CA, USA) at 37°C with shaking. *L. lactis* strain NZ9000 was cultured in M17 broth (Becton Dickinson/Difco, 7 Loveton Circle, Sparks, MD, USA) containing 5% glucose and incubated statically at 30°C. The antibiotic concentrations used to select the recombinant bacteria on agar medium were 100 μg/mL of ampicillin for *E. coli* or 5 μg/mL of chloramphenicol for *L. lactis*.

DNA cloning

All DNA cloning was performed as previously described (Sambrook and Rusell, 2001). The secretion plasmid pSEC:Nuc was kindly provided by Dr. Philip Langella (Bermudez-Humaran et al., 2004), and V3 loop were amplified from pSFV-HxB2 kindly provided by Dr. Bernard Verrier (Dacheux et al., 2004), A2B subunit from El Tor, variant of *Vibrio cholera* was amplified and kindly provided by Microbiology Laboratory of Biological Sciences, UANL, Mexico. To generate recombinant *L. lactis* pSEC:V3 and pSEC:A2B, the gene fragments for the V3 loop from gp120 of HIV-1 subtype B and the A2B subunit from CT were generated using PCR. The oligonucleotide sequences for the amplification of the V3 loop were 5'- GCG ATG CATCCT GTA CAA GAC CCAAC - 3' and 5'- GCC ACT AGT CC ACA ATG TGC TTG TCT - 3' and the sequences for the amplification of the A2B subunit were 5'- CCG ATG CAT CCA GTA ATA CTT GCGAT - 3' and 5'- CGG ACT AGT ATT TGC CAT ACT - 3'. Each forward primer contained an NsiI site in the 5' region, and each reverse primer contained a SpeI site in the 3' region. PCR reactions were performed in a total volume of 50 μL in a cocktail containing 2X Taq polymerase master mix (Promega, Madison, WI, USA), 200 ng of template DNA and 10 pmol of each primer. The amplification was performed with 35 cycles at 94°C for 1 min, 62°C for 30 s, and 72°C for 2 min. The resulting 115- or 510-kb PCR products were cloned into the pGEM-T vector (Promega, Madison, WI, USA) and transformed into *E. coli.* The PCR products cloned were digested, extracted from an agarose gel and purified using QIAEX II kit (Qiagen, Hamburg GmbH, Germany) prior to insertion downstream of the nisin-inducible promoter of the pSEC vector. To obtain the fusion plasmid pSEC:V3A2B, and pSEC:V3V3A2B the V3 loop was inserted into the plasmid pSEC:A2B upstream of A2B using the NsiI site (Table 1). All the constructs were characterized by enzymatic digestion and PCR, after that they were sequenced and electroporated into *L. lactis* NZ9000 electrocompetent cells with a single pulse of 2,500 V in a 0.4-cm electroporation cuvette using a Micropulser (BIO-RAD, Hercules, CA, USA). After that, *L. lactis* cells were incubated for 2 h at 30°C and plated on GM17 agar with chloramphenicol. The transformants were visible after 48 h of incubation.

Detection of V3 or A2B expressed in *L. lactis* recombinants by Western blots

The *L. lactis* recombinant proteins were induced using 1 ng/mL of nisin for 2 h at 30°C, and the supernatants and pellets were separated by centrifugation at 8,000 xg for 2 min. The pellets from 1 mL of recombinant bacteria were suspended in TES buffer (25% sucrose, 1 mM EDTA, 50 mMTris-HCl pH 8.0 and 10 mg/mL lysozyme) with 1 mM of phenylmethyl-sulfonylfluoride (PMSF) and 1 mM dithiothreitol (DTT). The sample was incubated for 30 min at 37°C prior to the addition of 50 μL of 20% SDS and soul of loading buffer. The supernatants were precipitated with trichloroacetic acid (TCA) and incubated on ice for 15 min. The pellets were obtained by centrifugation at 8,000 xg for 15 min at 4°C and were resuspended in 50 μL of 50 mM NaOH and 50 μL of loading buffer (100 mMTris-HCl pH 6.8, 4% SDS, 10% glycerol, 200 mM DTT and 0.1% bromophenol blue). The samples were separated by electrophoresis on a 15% sodium dodecyl sulfate-polyacrylamide

Table 1. Bacterial strains and plasmid used in this work.

Strain or plasmid	Relevant genotype or phenotype	Source/reference
L. lactis NZ9000	Wild type, MG1363 (nisRK genes in chromosome), plasmid free	Langella et al. (2004)
E. coli DH5 alpha	Wild type, *fhu*A2, Δ(argF-*lac*Z), U169, *pho*A, *gln*V44, φ80, Δ(*lac*Z)M15, *gyr*A96, *rec*A1, *rel*A1, *end*A1, *thi*-1, *hsd*R17.	Promega
Vibrio cholera 01 El Tor		Microbiology Department,UANL
pSFV-HxB2	Amp^r , SP6 promoter, encoding the envelope glycoproteins of HIV	Bernard Verrier et al. (2004)
pGEM-T	Amp^r	Promega
pSEC:Nuc	Cm^r, gene expressed from PnisA encodes SPUsp-Nuclease	Langella et al. (2004)
pSEC:A2B	Cm^r, gene expressed from PnisA encodes SPUsp-A2B subunit CT	This work
pSEC:V3	Cm^r, gene expressed from PnisA encodes SPUsp-V3 loop HIV	This work
pSEC:*V3*A2B	Cm^r, gene expressed from PnisA encodes SPUsp-V3A2B fused	This work
pSEC:*V3V3*A2B	Cm^r ,gene expressed from PnisA encodes SPUsp-V3V3A2B fused	This work

gel electrophoresis, transferred to Hybond membranes (Amersham, Buckinghamshire, England), blocked in a 5% solution of skim milk powder in Tris-buffered saline containing 0.05% Tween 20 and incubated at room temperature with shaking for 1 h. The membrane was allowed to react overnight at 4°C with a mouse monoclonal anti-V3 (ImmunoDx, Woburn MA, USA) or anti-A2B (AbDSerotec Raleigh, USA) antibody at a 1:1,000 dilution in blocking buffer and then with anti-mouse antibody conjugated to HRP (Sigma Aldrich, USA). Protein detection was performed using the chemiluminescent Super Signal West Pico ECL Substrate (Pierce, Rockford, IL, USA).

Immunization schedule

A total of 7 groups (5 mice per group) of 6-week-old female BALB/c mice were used for this study and immunized through oral administration with a single dose of 10^9 CFU live recombinant *L. lactis* that express V3, A2B, V3A2B, V3V3A2B or the combination of V3 and A2B. *L. lactis* NZ9000 or PBS were used as a negative control. For oral immunization, 10^9 UFC *L. lactis* recombinant or the control were suspended in 200 μL of PBS and intragastrically administered to the mice, according to Xu and Li (2007). The mice were sacrificed at ten days after immunization.

Sample collection

Samples of whole blood were collected by heart puncture and centrifuged at 600 xg for 10 min to obtain the serum. The intestinal washes were collected using 2 mL of TBS with PMSF, and then centrifuged at 600 xg for 10 min. The supernatant was stored at -20°C until analysis. PBMC were collected from whole blood heparinized by the method of Ficoll-Paque™ PLUS (GE Health care Bio-sciences AB, Sweden) employed according to the instructions of manufacturer.

Detection of HIV-specific antibodies anti-V3 IgG or IgA by ELISA

IgG or IgA antibodies against the V3 were detected in the serum and the intestinal wash samples by ELISA as follows: 5 μg of the V3 peptide RIQRGPGRAFVTIGK (Dalton Chemical Laboratories, Ontario, Canada) was directly coated onto the wells of a microtiter plate and incubated overnight at 4°C. The excess was removed, and the wells were washed three times with TBS. The wells were then blocked using 150 μL of TBS containing 3% BSA for 2 h at 37°C and washed as before. Each microplate well was incubated for 2 h with the sample at dilutions of 1:100, 1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400 or 1:12,800. After an additional extensive washing step, the microplate was incubated for 2 h with an anti-mouse IgG-HRP conjugate at a final dilution of 1:8,000 or an anti-mouse IgA conjugated with alkaline phosphate (Sigma Aldrich, USA). Finally, the plate was washed three times with TBS and ABTS (2,2' azino-di-(3-ethylbenzthiozoline sulfonic acid)) or pNPP (p-nitrophenylphosphate) at 2 mg/mL (Sigma Aldrich,USA) was added as substrate. The absorbance in each well was measured at 405 nm using an ELISA microplate reader (Synergy HT, BioTek. Winooski, VT, USA).

Detection of IL-2 cytokine by ELISA

PBMC cells from mice immunized orally with *L. lactis* recombinants were adjust to $1x10^6$ cells with RPMI (Invitrogen, Carlsbod, CA, USA) in 12 wells plate and sensitized with 5 µg of V3 peptide RIQRGPGRAFVTIGK. *L. lactis* NZ9000 and phytohemagglutinin (PHA) were used as a control. After 36 h of incubation at 37°C and CO_2 at 5%, supernatants were recover and IL-2 cytokine induced was detected by ELISA KIT (PEPROTECH, Rocky Hill, New Jersey, USA) following the manufacturers recommendations.

Statistical analysis

Results were representative data from at least three independent experiments. Mean data are presented with standard deviation and shown as error bars in the graphs. *P* values were calculated from ANOVA-Tukey´s HSD test. $P < 0.05$ was interpreted as being statistically significant.

RESULTS

Construction of plasmids for the expression of the V3 loop and A2B subunit of CT

The genes for the V3 loop and the A2B subunit of the CT were amplified by PCR, to created pSEC: V3 and pSEC:A2B, and their fusions pSEC:V3A2B, pSEC:V3V3A2B. All the constructs were characterized

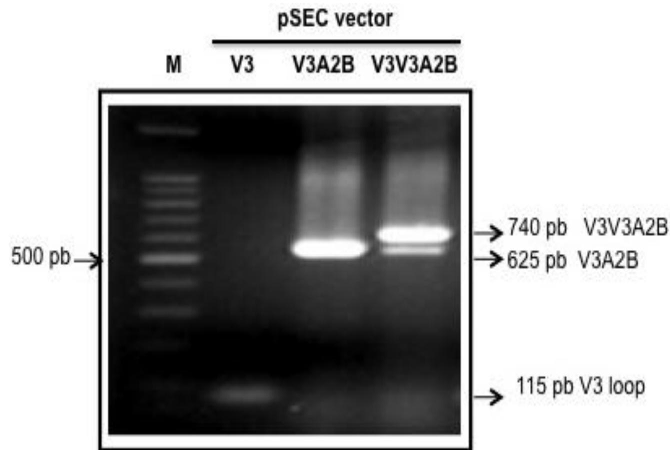

Figure 1. PCR analysis of the V3 loop gene fused to cholera toxin subunit B and cloned into pSEC vector. Lane M: MW DNA marker 100 pb, lane 1: pSEC:V3 vector, lane 2: pSEC:V3A2B vector, lane 3: pSEC:V3V3A2B vector.

using enzymatic restriction (data not shown), PCR analysis, fusions were characterized using V3 loop as forward and A2B as reverse oligonucleotides (Figure 1) and sequenced (data not shown), prior to electroporation into *L. lactis* NZ9000 competent cells.

Detection of expression of *L. lactis* recombinants proteins by Western Blot

The nisin-induced proteins from the pellet and the supernatants of each *L. lactis* strain were analyzed using Western blots. The recombinant proteins were detected only in the pellets (Figure 2). The recombinant *L. lactis*: V3*, L. lactis* V3A2B and *L. lactis* V3V3A2B were detected using an anti-V3 monoclonal antibody to ensure that the small protein would be recognized (Figure 2A, C and D,

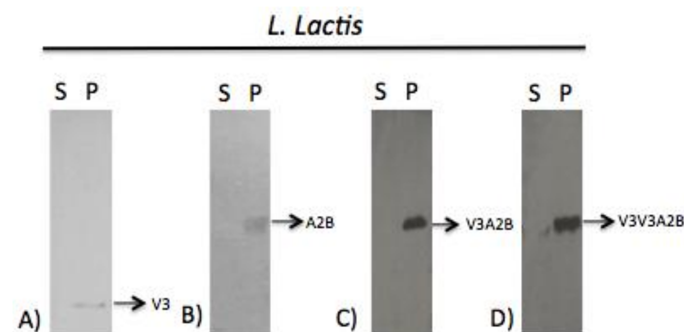

Figure 2. Detection of V3 loop, A2B subunit of cholera toxin and their fusions by Western Blot. Recombinant proteins by *L. lactis*. Supernatants (S) and pellets (P) of the recombinant bacteria were separated on a 15% SDS-PAGE gel, transferred to nitrocellulose and visualized via the chemiluminescence of an anti-V3 antibody used for the detection of the V3 loop (A), the V3A2B fusion (C) and the V3V3A2B fusion (D). A mouse anti-CT subunit B antibody was used to detect the A2B subunit (B).

respectively) and the recombinant *L. Lactis*:A2B were detected by anti-A2B monoclonal antibody (Figure 2B).

Anti-V3 IgG and IgA antibodies titers in the serum and intestinal washes

To analyze the humoral response induced by the different recombinant clones of *L. lactis*, we obtained sera and intestinal washes from the orally immunized mice. In orally immunized mice with *L. lactis* V3, *L. lactis* V3A2B, *L. lactis* V3V3A2B and the combination of *L. lactis* V3 and *L. lactis* A2B induced significant ($p<0.05$) anti-V3 IgA antibodies production in serum but not IgG, only *Lactis* V3V3A2B induced significant titers of anti-V3 IgG as compared to control *L. lactis* NZ9000 (Figure 3). All the recombinants, except A2B were efficient to induce significant ($p<0.05$) titers of anti-V3 IgA and IgG in intestinal washes, the most efficient was *Lactis* V3V3A2B as compared to the control NZ 9000 (Figure 4).

Analysis of IL-2 in PBMC by ELISA

To analyze the cellular response induced by the different recombinant clones of *L. lactis*, we obtained PBMC from the orally immunized mice. The recombinant *L. lactis* that express one loop V3 or their fusion to A2B of CT does not induce a significant ($p<0.05$) production of IL-2, only the recombinant *L. lactis* V3V3A2B that express two loops V3, was efficient to induce a significant production of IL-2 in PBMC (Figure 5).

DISCUSSION

Many strategies have been used to develop a HIV vaccine, recently, several replication-competent viral vectors based on vesicular stomatitis virus, vaccinia virus, measles virus and sendai virus were used in clinical trials. Viral vectors have proven to be well tolerated and immunogenic with evidence of efficacy in macaques (Parks et al., 2013). Another strategy is the bacterial vectors used to express HIV antigens to induce immunity (Charbit et al., 1993; Sirois et al., 2005). In this work, we used successfully, a non-pathogenic and non-colonizing candidate for delivering proteins by mucosal routes (Wang et al., 2008; Enouf et al., 2001)*: L. lactis,* to express the V3 loop from gp120 of HIV-1, or the A2B subunit from CT as an adjuvant, expressed before by Sun et al. (2009) and their different fusions are showed in (Table 1). Our recombinant proteins were not detected in the supernatants of the recombinant *L. lactis* strains, probably by an inefficient secretion of the protein, or the method used to detected it, but have the potential to induce a significant production of antibodies and cytokines *in vivo*. Similar results were found by Zhang et al. (2011) in hepatitis B expressed in *Lactococcus* on antibodies production, and supported by several reports

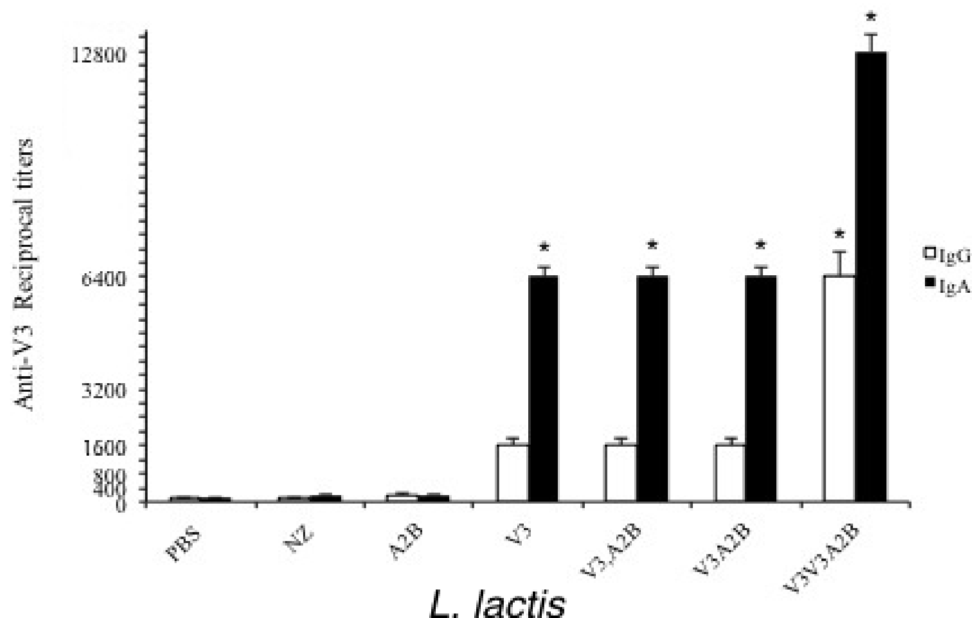

Figure 3. Titers of anti-V3 IgG and IgA antibodies in serum of mice immunized orally with 10^9 CFU live *L. lactis.* Serum from mice immunized with the recombinant that express loop V3 (V3), A2B Subunit CT (A2B), the combination of both (V3, A2B), and their fusion with one or two V3 loops (V3A2B), (V3V3A2B) or PBS or *L. lactis* wild type NZ9000 (NZ) as a negative control. Using an ELISA plate coated with 5 µg of V3 peptide in PBS, anti-V3 IgG and IgA antibodies were detected in serially diluted samples (1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400 and 1:12,800) of serum. (*) P<0.05 as compared to control NZ.

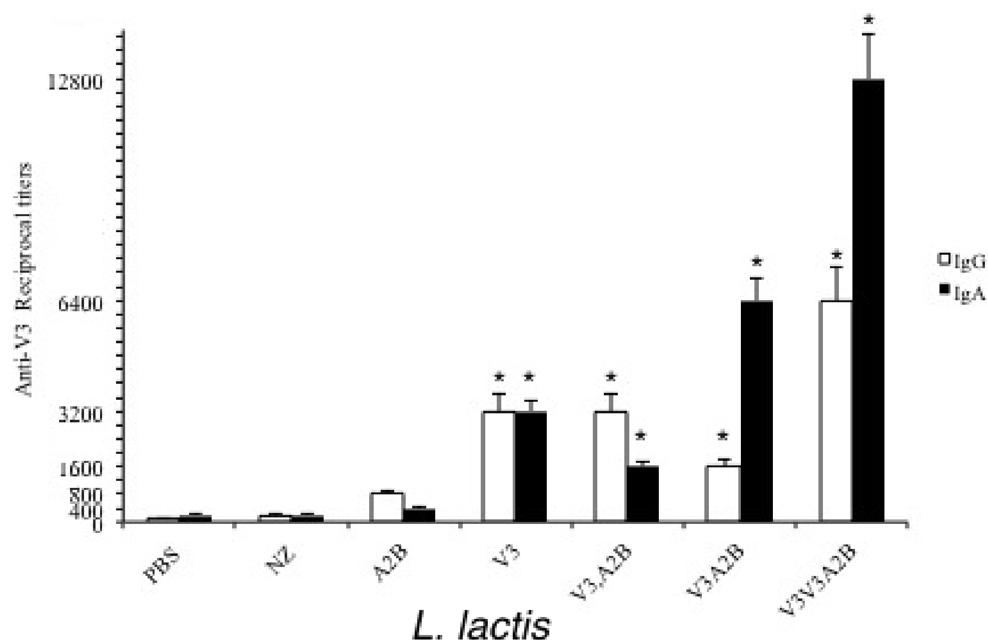

Figure 4. Titers of anti-V3 IgG and IgA antibodies in intestinal washes from mice immunized orally with 10^9 CFU live *L. lactis.* Intestinal washes from mice immunized with the recombinant that express loop V3 (V3), A2B Subunit CT (A2B), the combination of both (V3, A2B), and their fusion with one or two V3 loops (V3A2B), (V3V3A2B) or PBS or *L. lactis* wild type NZ9000 (NZ) as a negative control. Using an ELISA plate coated with 5 µg of V3 peptide in PBS, anti-V3 IgG and IgA antibodies were detected in serially diluted samples (1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400 and 1:12,800) of intestinal washes. (*) P<0.05 as compared to the control NZ.

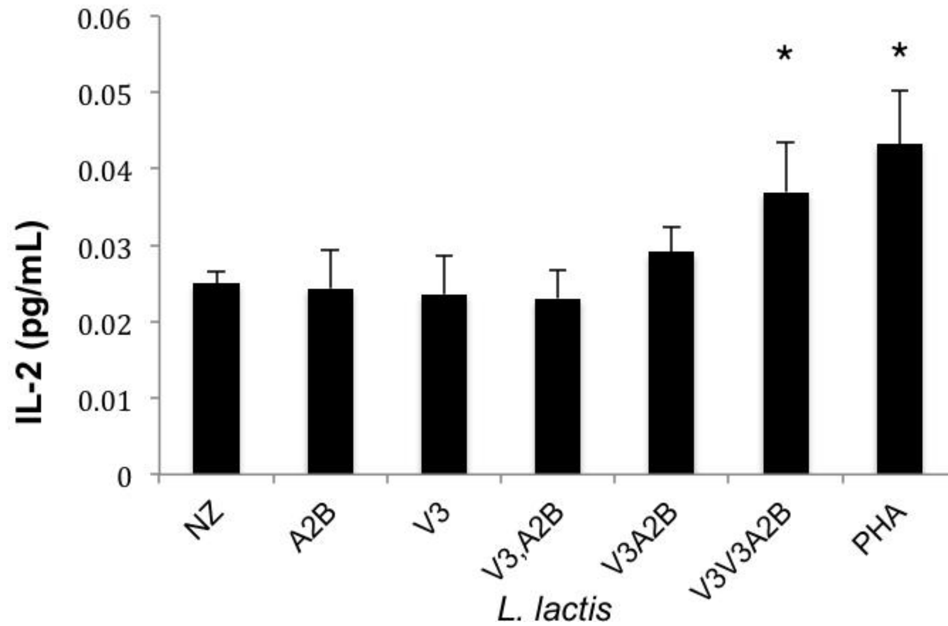

Figure 5. Analysis of IL-2 production by PBCM of mice immunized orally. Cytokine production of IL-2 by PBMC of mice immunized with 10^9 CFU live *L. lactis* wild type NZ9000 (NZ) as a negative control, or the recombinant that express loop V3 (V3), A2B Subunit CT (A2B) , the combination of both (V3, A2B), and their fusion with one or two V3 loops (V3A2B), (V3V3A2B). PHA was used as a positive control. (*) $P<0.05$ as compared to the control NZ.

of the induction of humoral immunity using intracellular protein expression in lactic acid bacteria (Grangette et al., 2001; Shaw et al., 2000; Su-Jung et al., 2006). We found that the expression of the V3 loop protein alone or fused to A2B efficiently induced anti-V3 antibodies IgA or IgG as compared to the control (*L. lactis* wild type, NZ9000); these results correlate with Xin et al. (2003) and Siroiset al. (2005), indicating that the V3 loop is an effective antigen for inducing humoral immunity against antigen HIV.

An induction of anti-V3 IgA or IgG antibodies as compared to the control (NZ 9000) occurred when *L. lactis* recombinant express V3 loop and A2B subunit CT. It has been reported that CTB induces a high titer of IgA antibody to HIV-1 (Bukawa et al., 1995; Kang et al., 2003) and IgG antibodies are induced less efficiently (Kim et al., 2004), like our results the IgG in serum were not significant as compared to IgA antibodies production.

The double fusion V3V3A2B was the most efficient statistically to induce anti-V3 IgG, IgA and IL-2 cytokine production as compared to other *L. lactis* recombinants used. It has been reported that CTB induce a better adjuvant effect (Albu et al., 2003; Seder et al., 1993).

It is important to note that specific antibodies production with one dose of recombinant bacteria was obtained, similar to that obtained in a model of gp120 expression by our laboratory (unpublished); however other studies required several doses of viral antigens expressed in lactic bacteria.

We fused two loop V3 with the purpose of increasing its immunogenicity, because V3 loop is small peptide as compared to A2B; our results determine that our strategy to increase the antigenicity was performed with double fusion V3V3A2B being the most efficient to induce a specific anti-V3 IgA or IgG antibodies and IL-2 production. We hypothesized that this effect probably is because the second V3 loop could function as a bridge between the first V3 loop and the A2B subunit, resulting in better exposure of the V3 loop for the induction of an immune response by antigen capture and presentation as well as improved protein stability. This study may provide an innovative and potent strategy of immunization to induce a specific immune response against HIV antigen in the mucosa, the main site of viral entry, greater than 90% of global HIV-1 transmission occurs across a mucosal surface (Griffin et al., 1996) through sexual transmission or mother to child transmission by breastfeeding, however it should be mentioned that the virus also could be transmitted by mechanisms where the mucosa is not involved, such as through the blood in drug users and donors in lesser proportion. Finally, an important aspect to consider in the use of live vector vaccines is the risk arising from the nature of the organism, mutations that cause a reversal and produce disease in the host, especially in immunocompromised patients, where the immune system determines the magnitude of risk. Although *Lactococcus* is a micro-organism that does not colonize, it could represent a risk

because the recombinants are usually selected by resistance to antibiotics and plasmid may transform the patient's microflora and spread resistance genes. More experiments on the capacity to recognize and neutralize a broad range of antibodies and T cell specific response against HIV should be done and finally corroborate these results in a primate model to determine efficacy.

Conclusions

We found that *L. lactis* may be used as an effective vaccine vector, and the strategy of fusing the V3 loop to an adjuvant, as the A2B subunit of the CT, could increase the immunity specific against HIV antigen. This study may provide an innovative and potent strategy of immunization to induce a specific immune response against HIV. Therefore, additional *in vivo* experiments using these recombinant *L. lactis* strains are necessary to determine whether the induced antibodies have a neutralizing capacity and whether this strategy may be used as an effective vaccine vector.

Conflict of interest

The authors declare that they have no conflict of interests.

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Full Length Research Paper

Comparative analysis of anti-*Staphylococcus aureus* action of leaves and fruits of *Anadenanthera colubrina* var. *cebil* (Griseb.) Altschul

Daniel Rodrigo Cavalcante de Araújo[1], Luís Claudio Nascimento da Silva[1]*, Alexandre Gomes da Silva[1], Janete Magali de Araújo[2], Alexandre José Macêdo[3], Maria Tereza dos Santos Correia[1] and Márcia Vanusa da Silva[1]

[1]Departmento de Bioquímica, Laboratório de Produtos Naturais, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil.
[2]Departmento de Antibióticos, Laboratório de Genética de Microorganismos, Universidade Federal de Pernambuco, Recife, Pernambuco, Brazil.
[3]Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

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This study evaluates the antimicrobial activity against *Staphylococcus aureus* and phytochemical constituents of leaves and fruits of *Anadenanthera colubrina* var. *cebil*. the hemolytic action of active fractions was also checked. After a liquid-liquid partition of hydroalcoholic extracts, the following fractions were obtained: cyclohexane (CFL), ethyl acetate (EAFL), n-butanol (NBFL) and aqueous fractions (AFL) from leaves; and cyclohexane (CFF), ethyl acetate (EAFF), n-butanol (NBFF) and aqueous fractions from fruits (AFF). The antimicrobial action of these samples was evaluated by microdilution assay against seven clinical isolates of *S. aureus* and the standard strain. Finally, the hemolytic activity of active fractions was checked. Phytochemical analysis detected the presence of triterpene, carbohydrate (leaves); flavonoids and tannin (fruit and leaves). All samples showed anti-*S. aureus* action and the most active fractions were EAL and EAF. The MIC ranged from 0.78125 to 6.25 mg/mL (EAFL) and 0.390625 to 1.5625 mg/mL (EAFL). Both fractions showed low toxicity since the HC50 values were greater than MIC50 values: the ratio MIC50/HC50 for EAFL was 3.68 and 11.61 for EAFF. Our work shows that *A. colubrina* is a potential source of anti-*S. aureus* molecules, their isolation and characterization are target of new research of our group.

Key words: *Anadenanthera colubrina,* caatinga, antimicrobial activity, hemolytic activity.

INTRODUCTION

The discovery of antibiotics in the 1930's revolutionized medicine in many respects, completely change the scenario of treatment of infectious diseases. However, 80 years later, we are at a critical point: new drugs are not

*Corresponding author. E-mail: luisclaudionsilva@yahoo.com.br.

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being developed at the pace necessary to stay ahead of the natural ability of bacteria to evolve and defend themselves against antibiotics (WHO, 2012; Rodríguez-Roja et al., 2013).

In the face of this problem, the search for bioactive molecules of natural origin has intensified, and molecular structures of secondary metabolism compounds have been elucidated, such as the class of flavonoids, tannins and other polyphenols with antibacterial, antifungal and antiviral properties (Cushnie et al., 2005; Newman and Cragg, 2012; Daglia, 2012). Various studies have shown that the synthesis of these metabolites is mediated by factors such as seasonality, availability of water, ultraviolet radiation, temperature, altitude, etc (Kroymann, 2011). The Caatinga biome is seen as an attractive source of bioactive molecules, as it presents extreme variations in these factors and it has a unique plant formation. This biome is located in northeastern Brazil, extending from 2°54’ to 17°21’S and its size is estimated at 800,000 km^2. As compared to other Brazilian ecosystens, the Caatinga has many extreme characteristics among climatic patterns: the highest solar radiation, low cloud cover, the highest annual average temperature, the lowest rates of relative humidity, higher potential evapotranspiration and, above all, precipitation limited to a very short period of the year (Mello et al., 2012).

One of the species found in this biome is *Anadenanthera colubrina* var. *cebil* (Leguminosae - Mimosoideae) which occurs mainly in seasonal forests, distributed in northeastern Brazil (Maranhão to Minas Gerais) in Caatinga as well as in southern Brazil in seasonal forests within the basins of the Paraguay and Paraná Rivers. It also occurs in the northeastern and northwestern Argentina, southeastern and southwestern Bolivia and the southern region of Ecuador (Soldati and Albuquerque, 2010).

The most recent analysis of this species was made by Alstchull (1964), in which *A. colubrina* var. *cebil* (Griseb.) Alstchul was described. Other names have been reported, such as: *Acacia cebil* Griseb., Goett. Abh. 19:136.1874; *Peptadenia macrocarpa* Benth., J. Bot. (Hooker) 4:341.1842; *P. macrocarpa* var. *cebil* (Griseb.) Chodart & Haasl., Bull. Herb. Boiss. ser. 2, 4:560. 1904; *Anadenanthera macrocarpa* (Benth.) Brenan, Kew Bull. 10:182.1955 (Queiroz, 2009). *A. colubrina* var. *cebil* is popularly known as *angico*, *angico-preto*, *angico-verdadeiro*, *angico-jacaré*, *angico-de-caroço* and *angico vermelho* (Queiroz, 2009). Bark of *A. colubrina* var. *cebil* (Griseb) Alstchull is used for the treatment of bronchitis; the fruits can be used as hallucinogens; while the leaves have shown no evidence of medicinal use (Agra et al., 2008).

Previous studies of crude extracts of *angico* have been restricted to the bark due to its wide popular use (Table 1) (Palmeira et al., 2010; Pessoa et al., 2012). Our group was the first to find evidence for the antimicrobial and antioxidant activity of crude extracts of the fruit of *A. colubrina* (Da Silva et al., 2011, 2013a, b; Silva et al., 2012).

Our group was also the first to analyze the inhibition of bacterial biofilms with leaves, fruit and bark of *A. colubrina*, finding the most significant activity from the bark (Trentin et al., 2013). In this context, the present study aims to analyze the phytochemical profile of the crude extract of leaves and fruits of *A. colubrina* var. *cebil,* as well as elucidate the antimicrobial potential of fractions from both extracts and the hemolytic effects of active fractions.

MATERIALS AND METHODS

Plant collection and plant storage

Leaves and fruits of *A. colubrina* var. *cebil* were collected in Parque Nacional do Catimbau, Pernambuco, Brazil, northeastern Brazil, in September 2010. Botanical identification was made by the staff of the Herbarium of Instituto de Pesquisa Agronômica de Pernambuco (IPA), Brazil, and voucher specimens were deposited in the herbarium (IPA 84.039). Leaves and fruits were dried at room temperature. The dried plants were milled to a fine powder in a Macsalab Mill (Model 200 LAB, Eriez®, Bramley), and stored at room temperature in closed containers in the dark until used.

Preparation of the crude hydroalcoholic extract

Leaves and fruits of *A. colubrina* var. *cebil* were dried at room temperature for 7 days, ground into a fine powder and used for extraction. The powder (20 g) from each tissue (fruit or leaves) was mixed with 50 mL ethanol : water (7:3) and submitted to agitation for 15 h. Then the extracts were filtered and the powder residue was mixed again with 50 mL ethanol-water and the entire extraction process was repeated. The supernatants collected were mixed in a round bottom flask and concentrated at 45°C (Silva et al., 2011). The residue was dissolved in dimethyl sulfoxide (DMSO) and kept at -20°C until use.

Phytochemical analysis

The phytochemical tests to detect the presence of tannins, flavonoids, anthocyanins, saponins, coumarins, quinones, anthraquinones, reducing compounds and alkaloids in each tissue (fruit or leaves) were performed according to the method described by Kokate (1994) and Harborne (1998).

Fractionation of the hydroalcoholic extract

The hydroalcoholic extract was dissolved in water, producing a solution that was submitted to successive liquid-liquid partitions with cyclohexane, ethyl acetate and n-butanol. The solutions produced were dried in anhydrous Na_2SO_4 and submitted to filtration under reduced pressure. Thereafter, the solvents were evaporated under reduced pressure in a rotary evaporator oven at 60°C, producing hexane, ethyl acetate and n-butanol phases (Oliveira et al., 2012). The residues obtained were kept at -20°C for future use.

Microbial strains

The antimicrobial activity of *A. colubrina* var. *cebil* leaves and fruit extracts and fractions was tested against the following

Table 1. Pharmacological potential of *Anadenanthera colubrina*.

Tissue	Traditional uses	Scientific account	Related compounds
Leaves or aerial parts	Anemia, lung inflammation, inflammations in general, cancer, blood thinner and other (Albuquerque et al., 2007).	Inhibitory activity on human platelet 12 -lipoxygenase, human reticulocyte 15-lipoxygenase and soybean lipoxygenase-1 (Gutierrez-Lugo et al., 2004).	Anadanthoflavone and other eleven know compunds. Anadanthoflavone was found to be active against 12-lipoxygenase (Gutierrez-Lugo et al., 2004).
Fruits	As narcotic and poison (Agra et al., 2008).	Larvicidal activity of seeds aqueous extract (Farias et al., 2010).	Farias et al. (2010) did not isolate compounds.
		Hydroalcoholic extract from fruits: Antioxidant activity and DNA protection capacities, inhibitory and synergetic effects with erythromycin against *S. aureus* (Da Silva et al., 2011, 2013a, 2013b).	Quercetin and Rutin were detected in hydroalcoholic extract from fruits.
Bark	Treatment of coughs, whooping coughs and bronchitis (Agra et al., 2008), lung inflammation, inflammations in general, cancer, blood thinner and other (Albuquerque et al., 2007).	Gum exudates: immunomodulatory and antitumor activities (Moretão et al., 2003, 2004).	ARAGAL, A complex acidic heteropolysaccharide, containing mainly galactose and arabinose residues.
		Healing activity of alcoholic extract (Pessoa et al., 2012).	Probable compounds:quercetin glycosides (Pessoa et al., 2012)
		Inhibition of *Pseudomonas aeroginosa* biofilm for aqueous extract and tannin fraction (Trentin et al., 2013)	Proanthocyanidins (or condensed tannins) were identified from *A. colubrina* fraction (Trentin et al., 2013)
		Anti-inflammatory and antinociceptive activities of aqueous extract (Santos et al., 2013).	Santos et al. (2013) did not isolate compounds.

Table 2. Microorganisms provided by the Departmento de Antibióticos (UFPEDA).

UFPEDA	Origin	Microorganism
100	ATCC	*Micrococcus luteus*
224	ATCC 25922	*Escherichia coli*
396	ATCC 29665	*Klebsiella pneumoniae*
86	ATCC 6633	*Bacillus subtilis*
02	ATCC 6538	*Staphylococcus aureus*
630	Nasal discharge	*Staphylococcus aureus*
663	Catheter Tip	*Staphylococcus aureus*
697	Secretion of chest drain	*Staphylococcus aureus*
679	Wound secretion	*Staphylococcus aureus*
709	Catheter Tip	*Staphylococcus aureus*
730	Nasal discharge	*Staphylococcus aureus*
733	Bone fragment	*Staphylococcus aureus*

microorganisms: *S. aureus* (UFPEDA02), and some recently isolated strains of *S. aureus* (UFPEDA 733, UFPEDA 730, UFPEDA 709, UFPEDA 679, UFPEDA 697, UFPEDA 663 and UFPEDA 630) (Table 2). All strains were provided by the Departamento de Antibióticos, Universidade Federal de Pernambuco (UFPEDA) and maintained in Müeller-Hinton agar (MHA) and stored at 4°C.

Determination of antimicrobial activities

Disc diffusion method

Briefly, a sample 100 µL of microbial suspension (1.5×10^8 CFU/mL) were spread in Petri plates containing MHA, and sterile paper discs (containing 2000 µg of extracts) were added. After 18 h of incubation, the diameter of the zone of growth inhibition was examined. Dimethyl sulfoxide was used as the negative control (Bauer et al., 1966).

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

MIC was determined by the microdilution method (CLSI, 2011). A two-fold, serial dilution of the extract/fractions was prepared in Müeller-Hinton Broth (MHB) and 100 µL (approximately 1.5×10^8 CFU/mL) of bacteria suspension was added. The sample initial concentration was 50 mg/mL. The samples were incubated for 24 h at 37°C. Resazurin solution (0.01%) was used as an indicator by color change visualization: any color changes from purple to pink were recorded as bacterial growth. The lowest concentration at which no color change occurred was taken as the MIC. The MIC50 was defined as the MIC value capable of inhibiting 50% of the isolates. Afterwards, cultures were seeded in MHA medium and incubated for 24 h at 37°C to determine the MBC which corresponds to the minimum concentration of extract/fractions that eliminated the bacteria.

Table 3. Antimicrobial activity of hydroalcoholic extracts from *A. colubrina* leaves[1] and fruits[2].

Microrganism (UFPEDA)	Inhibition (mm)	Inhibition (mm)	MIC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MBC (mg/mL)
	HEL[1]	HEFr[2]	HEL [1]	HEFr[2]	HEL[1]	HEFr[2]
(100) *Micrococcus luteus*	N/A[b]	17.67±0.57[b]	N/A[b]	0.7812[b]	N/A[b]	3.125[b]
(224) *Escherichia coli*	N/A[b]	N/A[b]	N/A[b]	N/A[b]	N/A[b]	N/A[b]
(396) *Klebsiella pneumoniae*	N/A[b]	N/A[b]	N/A[b]	N/A[b]	N/A[b]	N/A[b]
(86) *Bacillus subtilis*	17.33 ±1.15[b]	13 ±1.73[b]	6.25[b]	1.5626[b]	12.5[b]	6.25[b]
(02) *Staphylococcus aureus*	14.67 ± 0.57[b]	14.67 ± 0.57[ba]	0.7812[a]	1.5626[ba]	12.5[b]	6.25[ba]

N/A: No activity; [a] values found for Da Silva et al. (2013a) and [b] Silva et al. (2012).

Table 4. Phytochemical analysis of *Anadenanthera colubrine* var. *cebil.*

Tissue	Compound						
	Steroid	Saponin	Alkaloid	Flavonoid	Tannin	Sugar	Terpenoid
Leaves	+	-	-	+	+	+	+
Fruits	+	-	-	+	+	-	+

***In vitro* hemolytic assay**

Blood (5 to 10 mL) was obtained from healthy non-smoking volunteers by venipuncture, after the obtaining a written informed consent. Human erythrocytes from citrated blood were immediately isolated by centrifugation at 1500 rpm for 10 min at 4°C. After removal of plasma and buffy coat, the erythrocytes were washed three times with phosphate-buffered saline (PBS; pH 7.4) and then resuspended using the same buffer and a 1% erythrocyte suspension was prepared. The hemolytic activity was tested under *in vitro* conditions. Each tube received 1.1 mL of erythrocyte suspension and 0.4 mL of extract of various concentrations (50 to 500 μg/mL). The negative control was only solvent and the positive control received 0.4 mL of *Quillaja* sp. saponin (0.0025%). After incubation of 60 min at room temperature, cells were centrifuged and the supernatant was used to measure the absorbance of the liberated hemoglobin at 540 nm. The average value was calculated from triplicate assays. The hemolytic activity was expressed in relation to saponin activity, and calculated by the following formula (Oliveira et al., 2012):

Hemolytic activity (%) = (As-Ab)×100/(Ac-Ab);

Where, Ab is the absorbance of the control (blank, without extract), As is the absorbance in the presence of the extract and Ac is the absorbance of the saponin solution.

Statistical analysis

Each experiment was performed in triplicate and results are expressed as the mean ± standard deviation (SD). Statistical analysis was performed by Student's t-test. Differences were considered significant at $p < 0.05$. The correlation indices were calculated using the Pearson coefficient (ρ). The concentration needed for 50% of hemolysis (HC50) was estimated using linear regression analysis.

RESULTS

This study provided a comparative analysis of the antimicrobial l activity of leaves and fruits of *A. colubrina* against *S. aureus*. Our previous works have demonstrated that crude hydroalcoholic of both tissue are active against Gram-positive bacteria, such as *S. aureus* (Table 3). To identify the groups of molecules that likely participate in this inhibition, phytochemical tests were performed and the presence of tannins, flavonoids, proanthocyanidins, triterpenes and sugars were verified in the crude leaf extract of *A. colubrina* var. *cebil*. In the crude extract of the fruit, it was possible to verify the presence of tannins and flavonoids (Table 4).

The following steps for a better elucidation of this activity involved semi-fractionation of crude extracts by the cold liquid-liquid method. The MIC values for the fractions derived from the leaf ranged from 25 to 0.781 mg/mL and the MBC ranged from 3.125 to >25 mg/mL against different strains of *S. aureus* (Table 5). Of the strains tested, 62.5% were sensitive to the cyclohexane fraction (CFL) at a concentration of 1.562 mg/mL, 87.5% were sensitive to the ethyl acetate fraction (EAFL) at concentration of ≤ 1.562 mg/mL, 57.1% were sensitive to a concentration of 0.781 mg/mL. The values of MBC were considered high in comparison with the MIC found and ranged from 3.125 to >25 mg/mL. The values of MIC and MBC of the n-butanol (NBFL) and aqueous fractions (AFL) increased dramatically showing MIC values of 3.125 to 25 mg/mL and MBC values of 6.25 to >25 mg/mL (Table 5). EAFL was the best fraction showing MIC values up to 16 times greater than other fractions and its MIC50 was 0.78 mg/mL.

As for the fractions derived from the fruit, all strains were sensitive to the ethyl acetate fraction (EAFF) at a concentration ≤ 1.562 mg/mL, of these 62.1% were sensitive to 0.39 mg/mL (EAFF). As for the MBC of EAFF only three strains were sensitive to a concentration of ≤3.125 mg/mL. In the case of the fractions derived from

Table 5. Antimicrobial activity of *A. colubrina* leaf fractions.

Microrganism	Cyclohexane fraction (CFL)		Ethyl acetate fraction (EAFL)		n-Butanol fraction (NBFL)		Aqueous fraction (AFL)	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
UFPEDA02	3.125	6.25	6.25	12.5	12.5	12.5	25	>25
UFPEDA630	6.25	12.5	0.78125	>25	N/A	N/A	12.5	12.5
UFPEDA663	1.5625	>25	0.78125	12.5	12.5	>25	25	>25
UFPEDA679	1.5625	>25	1.5625	12.5	25	>25	25	>25
UFPEDA697	1.5625	>25	1.5625	12.5	12.5	>25	25	>25
UFPEDA709	1.5625	3.125	0.78125	3.125	12.5	12.5	25	25
UFPEDA730	6.25	12.5	1.5625	25	N/A	N/A	N/A	N/A
UFPEDA733	1.5625	12.5	0.78125	12.5	12.5	12.5	25	25

Table 6. Antimicrobial activity of *A. colubrine* fruit fractions.

Microrganism	Cyclohexane Fraction (CFF)		Ethyl acetate fraction (EAFF)		n-Butanol fraction (NBFF)		Aqueous fraction (AFF)	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
UFPEDA02	12.5	12.5	1.5625	6.25	12.5	12.5	25	>25
UFPEDA630	25	>25	0.78125	1.5625	12.5	>25	12.5	>25
UFPEDA663	25	>25	0.78125	3.125	>25	>25	25	>25
UFPEDA679	25	>25	0.390625	12.5	12.5	>25	25	>25
UFPEDA697	25	>25	0.390625	6.25	12.5	>25	12.5	>25
UFPEDA709	12.5	>25	0.390625	12.5	3.125	>25	12.5	>25
UFPEDA730	>25	>25	0.390625	1.5625	3.125	6.25	25	25
UFPEDA733	25	>25	0.390625	6.25	12.5	25	25	>25

the fruit, the other fractions (cyclohexane, n-butanol and aqueous) presented values of MIC and MBC considered high, as compared to the ethyl acetate fraction, and ranged from 3.125 to 25 and 6.25 to >25 mg/mL, respectively (Table 6). The MIC50 for EAFF was 0.39 mg/mL.

Hemolytic activity is one of the analyses carried out to assess the cytotoxicity of a sample. The effects on viability of red blood cells of human origin were determined for the most active fractions of this study (ethyl acetate fractions). It was found that the HC50 were 2.874 and 4.534 mg/mL for EAFF and EAFL, respectively (Figure 1).

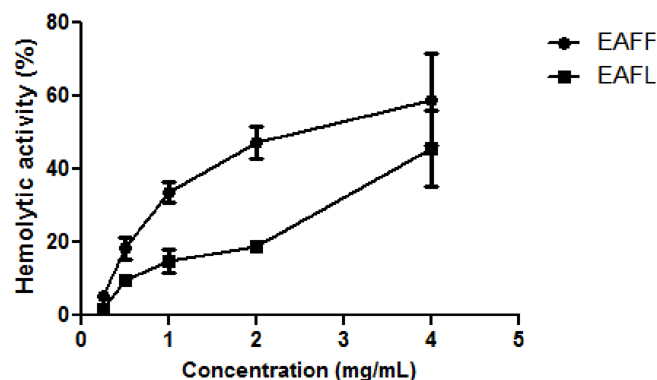

Figure 1. Hemolytic activity of ethyl acetate fractins from *A. colubrina* leaves (EAFL) and fruits (EAFF).

DISCUSSION

The choice of working with clinical strains of *S. aureus* was based on academic and medical concern. *S. aureus* is a commensal bacterium that colonizes both animal and human, which uses various strategies for persistence in the host, for example antibiotic resistance, release of virulence factors and switching to a small-colony variant phenotype (Du Toit, 2014; Powers and Wardenburg, 2014). Taken together, all these features result in the incidence of this pathogen among hospital-acquired infections (Davis et al., 2013) and make crucial the search of new anti-*S. aureus* compounds.

In this study, we demonstrated for the first time, the anti-

S. aureus activity of fractions obtained from hydroalcoholic extracts of *A. colubrine* leaves and fruits, the hemolytic action of active fractions and phytochemical analyses of these tissues. The inhibitory activity of hydroalcoholic extracts from *A. colubrina* leaves and fruits against *S. aureus* have been shown in various researches (Silva et al., 2012), including the synergism with erythromycin of hydroalcoholic extract from fruits (Da Silva et al., 2013b) and the involvement of cell wall damage in its action, which could cause the loss of viability and cell death (Da Silva et al., 2013a).

Firstly, the comparative investigation of phytochemical compounds presented in each tissue revealed that while leaves were formed by tannins, flavonoids, proanthocyanidins, triterpenes and sugars, in the fruits only tannins and flavonoids were detected. These results showed that all the tested fractions had antimicrobial activity at least for one strain, suggesting that all solvents are able to solubilize at least one kind of active compounds. However, the best results were found for ethyl acetate fractions of both tissues.This best potential of ethyl acetate fractions could be explained by its capacity to solubilize molecules with antimicrobial properties such as tannins, leucoanthocyanidins and some groups of flavonoids. In fact, tannins and flavonoids have been indicated as active molecules in *A. colubrina* (Gutierrez-Lugo et al., 2004; Trentin et al., 2013). In addition, a close relationship were found between tannin levels of *A. colubrina* and seasonal climate changes in the caatinga (Monteiro et al., 2006), as well its ethnopharmacology uses as anti-inflammatory and healing agent (Sousa Araújo et al., 2008).

Looking at the most active fractions of EAFL and EAFF in Tables 3 and 4 (smallest MIC value), we can say that they have a tendency towards bacteriostatic behavior (MBC/MIC >4), using the MBC/MIC ratio (Pankey and Sabath 2004). These fractions showed a strong correlation (ρ=0.84) in their MIC values, however, ELFF had the lowest MIC values (2-4 times) for most strains (75%).

Finally, the cellular toxicity of the most active fractions was evaluated using human erythrocytes as a test system. This assay is based on hemoglobin release caused by cell membrane damage and can also reveal some information on the involvement of the cytotoxicity mechanism (Hassan et al., 2010; Kalaivani et al., 2011). Both fractions showed low toxicity since the HC50 values were greater than MIC50 values: the ratio MIC50/HC50 for EAFL was 3.68 and 11.61 for EAFF. These results also confirmed that EAFF have the best potential as a source of antimicrobial agents.

Conclusion

In conclusion, these data demonstrated that leaves and fruits of *A. colubrina* var. *cebil* are potential sources of molecules against *S. aureus*. For both tissues, the best results were found for ethyl acetate fraction, being EAFF the most active due its high action and low hemotoxicity. The purification and structural characterization of active compounds of this plant is the next step of our research.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Correlation between inoculum volume, positivity rates and microorganisms isolated from blood cultures

Wadha A. Alfouzan[1,2]*, Fawaz Y. Azizieh[3] and Rita Dhar[1]

[1]Microbiology Unit, Department of Laboratories, Farwania Hospital, Kuwait.
[2]Department of Microbiology, Faculty of Medicine, Health Sciences Centre, Kuwait University, Kuwait.
[3]Department of Mathematics and Natural Sciences, Gulf University for Science and Technology, Kuwait.

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The study aimed to assess the volume of blood inoculum in each culture bottle and its effect on the rate and time to positivity, types of microorganisms isolated and contamination rate. During a period of six months, two sets of blood cultures (aerobic and anaerobic: 3006 bottles each) were collected. Of the bottles collected, only 2916 aerobic and 2898 anaerobic culture bottles were analysed by categorising the samples into three different blood volume groups: < 8 ml, 8-10 ml and >10 ml, where ≥8 ml is considered an adequate volume. In the aerobic bottles, there was no significant difference in the rate of positivity among the three groups: 8-10 ml and < 8 ml (p = 0.226) or > 10 ml (p = 0.282). However, there was a significant decrease in positivity in the anaerobic bottles when comparing the > 10 ml blood group with the < 8 ml group (p = 0.032). The contamination rate was lower in the > 10 ml volume category in aerobic blood cultures (p = 0.008) and the isolation rate of Gram-negative bacilli was higher in this category compared with the other two categories (p = 0.05). Our study shows that the volume of blood cultured was a variable that influences the positivity rate and contamination rate of a blood culture sample.

Key words: Blood culture, blood volume, positivity rates, bacterial isolates.

INTRODUCTION

Proper collection of blood cultures has been reported to be the cornerstone for the diagnosis of bacteraemia and sepsis. In addition, it also depends on the site where the blood is drawn from and on the volume of blood inoculated in the culture bottles (Connell et al., 2007; Gonsalves et al., 2009; Shafazand and Weinacker, 2002). Correct interpretation of the culture results is vital to initiate appropriate antimicrobial therapy in a patient, as contamination from skin flora is a common occurrence leading to misinterpretation of the results in certain clinical situations (Shafazand and Weinacker, 2002). Although the most common contaminants are coagulase-negative staphylococci (CoNS); in the majority of studies worldwide these organisms are considered as primary

*Corresponding author. E-mail: alfouzan.w@hsc.edu.kw. Tel: 965-24636516. Fax: 965-25332719.

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pathogens in immuno-compromised patients as well as in those with indwelling intravascular devices (Huebner and Goldman, 1999; Von Eiff et al., 2002). The uncertain clinical significance of isolation of potential contaminants may result in extended hospital stay, unnecessary antibiotic therapy and additional laboratory investigations. This leads to increased expenditures of 39% in antibiotic charges and 80% in microbiology laboratory charges (Bates and Goldman, 1991). By contrast, isolation of a potential pathogen or even exclusion of bacteraemia has a direct impact on the management of the patient, with inclusion of optimal antibiotic therapy in the former and cessation of unnecessary antibiotic use in the latter (Shafazand and Weinacker, 2002).

Among the many factors that are known to influence the yield of microorganisms from blood cultures, the single most important factor is the blood volume (Shafazand and Weinacker, 2002). Evidence from both adult and paediatric studies show that the rate of isolation from blood culture increases with the volume of blood collected (Jonsson et al., 1993). The ability to exclude bacteraemia on the basis of negative blood culture results depends on the sensitivity and on the negative predictive value of the test. The interpretation of negative blood culture results is based on studies using adequate volumes of blood in research settings. When the volume of blood submitted for culture is inadequate, the negative blood culture result is potentially misleading, as it can falsely exclude significant bacteraemia (Connell et al., 2007). Although the value of blood cultures in the management of septic patients has been well documented in several studies, there are recent reports that have questioned this practice, particularly in patients with pneumonia (Abe et al., 2009). This current study aimed to determine the correlation between inoculum size and its effect on the positivity rates, time to positivity and isolation rates of different microorganisms from blood cultures received in our laboratory at the Farwania Hospital. Furthermore, we looked at the effect of inoculum size in relation to contamination rates.

MATERIALS AND METHODS

Farwania Hospital is a general hospital with 1200 beds serving mainly an urban population. It houses medical and surgical specialties and subspecialties. However, cancer, HIV and burn patients are referred to specialised institutions. All blood cultures received from patients during a 6-month period (March to August, 2009) were analysed. As a routine practice, blood cultures were ordered as a set, which contained one aerobic (BACTEC Plus Aerobic/F) and one anaerobic bottle (BACTEC Plus Anaerobic/F) (Becton Dickinson Diagnostics, Sparks, MD, USA). A minimum of two sets of blood cultures, drawn at least one hour apart, were received in the laboratory. The total blood volume collected was assumed to have been distributed equally in the two bottles at the same time. These bottles require a minimum inoculum size of 5 ml, although ideally, 8-10 ml is recommended according to the manufacturer's instructions. Each inoculated bottle was weighed to two decimal places using a digital balance. The volume of blood in each bottle was calculated by subtracting the weight of the un-inoculated bottle from the weight of inoculated bottle as described previously (Bouza et al., 2007), and the density of blood was assumed to be 1 (Lingwood et al., 2006). All bottles were incubated at 35°C in asemi-automated BACTEC system (BD Diagnostics) and monitored for a positive signal over a five-day period. When the system detected growth in an individual blood culture bottle, the time to positivity was recorded and the broth was examined by microscopy and further cultured on appropriate agar plates. Of the 3006 sets of blood cultures received, 2916 aerobic and 2898 anaerobic blood cultures were analysed. Bottles for which the weight measurement or other data, such as patient's age or culture results were missing were excluded from analysis. True bacteraemia was considered when Gram-positive cocci, Gram-negative bacilli (GNB), and yeast were isolated from one or more blood cultures from the same patient. Coryneforms, *Bacillus*, *Micrococcus* spp. and non-pneumococcal α-haemolytic streptococci (except if multiple bottles were positive) were classified as contaminants. Additionally, CoNS were classified as contaminants if they grew out of only one blood culture with an extended time to positivity (> 24 h). However, they were considered to represent true bacteraemia if the same organism grew on multiple consecutive cultures. For the purpose of analysing the data, inoculated blood volumes in aerobic and anaerobic blood culture bottles were grouped into three categories based on blood volume (< 8 ml, 8-10 ml, and > 10 ml) on a similar pattern as previously described (Lingwood et al., 2006). Inadequate volume was regarded as < 8 ml and adequate volume was regarded as ≥8 ml. Furthermore, >10 ml was given a separate grouping entity to ascertain whether this increase in volume affects positivity and or contamination rates.

Statistical analysis

The data were analysed using the computer software Statistical Package for Social Sciences, Windows version 19.0 (SPSS, Chicago, IL, USA). The descriptive statistics are presented as the number and the percent of positive results. The Z-test was used to test whether any significant differences exist in the proportion of positive samples in the two groups as compared with the standard group, which was considered as the group with highest number of positive results. The odds ratio (OR) and 95% Confidence Interval (CI) were also calculated. A Chi-square 'χ^2' test was applied, as appropriate, to assess the statistical significance of the association between proportions. The two-tailed probability value ($p < 0.05$) was considered statistically significant.

RESULTS

Aerobic bottles

Volume

Of the 2916 aerobic cultures received in the 6-month period, 297 (10.2%) were positive, with 129 (43.4%) being in the inadequate volume category (<8 ml) and 168 (56.6%) in the adequate volume category (≥8 ml) as shown in Table 1 and Figure 1.

Time to positivity

Of the 297 positive blood cultures from the aerobic cultures, 224 (75.4%) signalled positive result within 24 h. There was no difference in the time to positivity rate

Table 1. Positivity rates among aerobic and anaerobic culture bottles in the three inoculated blood volume categories

Blood volume (ml)	Blood culture bottle							
	Aerobic (2916)				Anaerobic (2898)			
	Positive	Negative	% Positive	Positivity rate	Positive	Negative	% Positive	Positivity rate
< 8	129	1196	9.7	4.4	139	1508	8.4	4.8
8-10	80	611	11.6	2.7	55	511	9.7	1.9
> 10	88	812	9.8	3.0	43	642	6.3	1.5

% Positive = Number of positive cultures / total number of cultures in corresponding blood volume category. Positivity rate = number of positive cultures / total number of cultures in all blood volume categories.

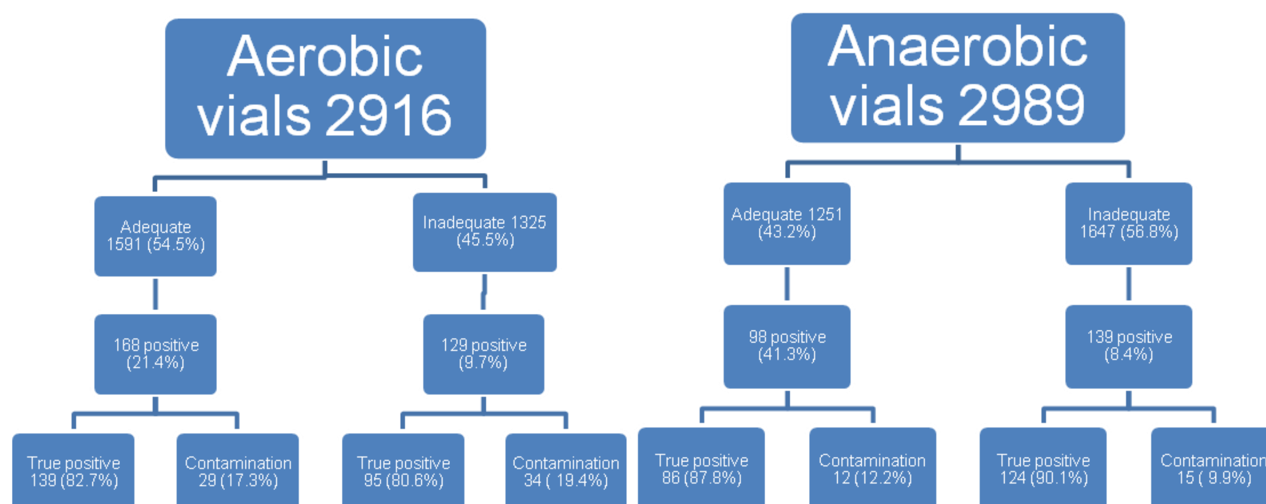

Figure 1. Distribution of blood cultures based on results and volumes.

between the adequate and inadequate blood volume cultures, even in the samples with > 10 ml blood volume.

Organisms isolated

There was no difference in the isolation rate of GNB (mainly members of *Enterobacteriaceae*, *Pseudomonas*, and *Acinetobacter* spp.), *S. aureus* and *Candida* spp. between the adequate 29 (17.3%) and inadequate 34 (19.4%) blood culture volume groups. However, the isolation rate of GNB was significantly higher in the > 10 ml category compared with the other two categories in the aerobic blood cultures (61.5%) (p = 0.021). In addition, the contamination rate (7.3%) was significantly lower in the > 10 ml category (p = 0.008) (Table 2).

Anaerobic bottles

Volume

Of the 2898 anaerobic cultures, 237 (8.2%) were positive and included facultative organisms, with 98 (41.3%) having adequate blood volume (Table 1 and Figure 1).

Time to positivity

Time to positivity was similar in both the adequate and inadequate blood volume cultures. However, 45 vials (81.8%) in the 8-10 ml blood volume category signalled positive in ≤ 24 h. Additionally, when the 8-10 ml category was compared with the < 8 ml and the > 10 ml categories, the resulting ORs were 2.5 (95% CI 1.14 to 5.3; p = 0.022) and 1.4 (95% CI 0.51 to 3.7; p = 0.54), respectively. A statistically significant difference (p < 0.01) was found when the samples that signalled positive within the first 24 h in the 8-10 ml volume category were compared with samples in the < 8 ml category signalling positive during a similar time period.

Organisms isolated

There was no statistically significant difference in the

Table 2. Microorganisms isolated from blood culture in each category.

Blood culture vial	Blood volume (ml)	Positive samples (n)	Microorganism[a] isolated number (%)*							
			Total[b] (n)	GNB	STREP	SAUR	CONS	CONT	CAND	ANAE
Aerobic	< 8	129	175	82 (46.9)	9 (5.1)	20 (11.4)	26 (14.9)	34 (19.4)	4 (2.3)	0
	8-10	80	94	47 (50.0)	6 (6.4)	5 (5.3)	12 (12.8)	22 ((23.4)	2 (2.1)	0
	> 10	88	96	59 (61.5)[c]	8 (8.3)	12 (12.5)	8 (8.3)	7 (7.3)[c]	2 (2.1)	0
Anaerobic	< 8	139	152	80 (52.6)	12 (7.9)	15 (9.9)	24 (15.8)	15 (9.9)	1 (0.7)	5 (3.3)
	8-10	55	59	31 (52.5)	6 (10.2)	7 (11.9)	6 (10.2)	8 (13.6)	0	1 (1.7)
	> 10	43	45	32 (71.1)[c]	2 (4.4)	4 (8.9)	2 (4.4) [c]	4 (8.9)	0	1 (2.2)

[a]GNB, Gram-negative bacilli; STREP-,*Streptococci* (including *S. pneumoniae*); SAUR,*S. aureus*; CONS, Coagulase-negative *Staphylococci*; CONT, contaminants; CAND, *Candida* spp.; ANAE, anaerobes, .[b] Includes mixed growth of microorganisms, [c] $p < 0.05$ (comparing yield from > 10 ml volume to other two categories); *No. of isolates / total number of microorganisms in respective category

isolation rate of other microorganisms from both blood culture categories. However, the isolation rate of GNB was significantly higher in the > 10 ml category (71.1%) ($p = 0.027$). Furthermore, in the >10 ml volume category, the isolation rate of CoNS (usually considered contaminants unless proven otherwise) was lower, with a value of 4.4% ($p = 0.05$). Concerning the total number of samples categorised as true anaerobes; it was 7 (2.9%), with 5 (71.4%) being in the inadequate volume category (Table 2).

DISCUSSION

In this study, the overall incidence of positive results of 9.2% was lower than the internationally reported rate of 21.5-24.5% (Gonsalves et al., 2009; Bouza et al., 2007). A probable explanation could be due to the increased risk of mortality in patients with bacteraemia, in which there is generally a lower threshold for drawing blood culture samples, which increased our denominator (Bates and Goldman, 1991). However, the contamination rate of 1.6% in this study was within the range of 0.6-6.0% shown in previous studies (Lingwood et al., 2006; Bekeris et al., 2005). Several factors are known to influence blood culture yield, including low rates of bacteraemia, low numbers of circulating colony-forming units, intermittent bacteraemia and antimicrobial properties of blood components. Other factors include clinical and laboratory variables, such as volume of blood drawn, concurrent antibiotic use, number of blood sample cultured, timing of blood collection, length of incubation of blood cultures, atmosphere of incubation and culture media and collecting system used (Shafazand and Weinacker, 2002; Darby et al., 1997). It is conceivable that because this study was laboratory-based, interplay of any of the aforementioned clinical barriers was unknown.

BACTEC systems are the most widely used blood culture systems in microbiology laboratories worldwide and have been reported to be superior to other blood culture systems, particularly for the isolation of *S. pneumoniae*, *Pseudomonas aeruginosa*, anaerobic bacteria and other *Streptococcus* spp. (Doern, 1994; Weinstein, 1996). Although the use of a combination of BACTEC and DuPont Isolator Systems can broaden the range of detection of bacterial and fungal infections, it is not economically feasible. For routine clinical conditions, the use of BACTEC systems and the duration of incubation of blood culture vials for five days have been found to be adequate (Pohlman et al., 1995). It is generally accepted that the yield of blood cultures from adults is volume dependent, with an increase of 3-5% in the detection rate with the addition of each millilitre of blood (Mermel and Maki, 1993; Tarrand et al., 1991).

It is established that most blood stream infection (BSI) episodes in adults have a low density of microorganisms in the blood with an average of 1 CFU/ml, necessitating greater volume of blood to be cultured in order to obtain a positive yield (Darby et al., 1997; Weinstein, 1996; Pohlman et al., 1995; Tarrand et al., 1991). However, our study shows that the increased yield in the > 10 ml category was observed for GNB only ($p < 0.05$), whereas there was no significant increase in the detection rate of other microorganisms (Gram-

positive bacteria and yeasts). Similar findings were reported in an earlier study (Weinstein et al., 1996), in which another continuous-monitoring blood culture system, the BacT/Alert system (Organon Teknika Corp, Durham, NC, USA) was used. The possible explanation for the higher yield of GNB could be shorter generation times (20 min) when compared with others.

However, our data indicated that the contamination rate was lower in the >10 ml category compared with the < 8 and 8-10 ml categories (*p*<0.008), which is in consistent with the finding of an inverse correlation between blood culture contamination and the volume of blood, previously reported by Bekeris et al. 2005 and Gonsalves et al., 2009. Although the authors did not present any obvious explanations, they hypothesised that during venipuncture, the contaminating skin microflora are likely to be diluted and are therefore less likely to be detected during the 5 days of incubation. In our study, the occurrence of positive samples in the aerobic and anaerobic bottles within 24 h of incubation in the BACTEC system was 75.4 and 70.9%, respectively. In an analogous study, the average detection time of growth was observed to be 13.55 h (range, 0.66-89.02 h) regardless of the blood volume inoculated in the culture bottles (Weinstein et al., 1996).

To shorten and improve current laboratory procedures for detecting microorganisms causing blood stream infection, an ideal diagnostic technique is being investigated to overcome limitations of culture. Several nucleic acid-based techniques have been developed for infection diagnosis and pathogen identification (Dark et al., 2009; Paolucci et al., 2010; Ecker et al., 2010).

Our study was limited by laboratory-based diagnosis and hence clinical interpretation of blood culture result is not included. However, it was clear that a blood volume of >10 ml contributes significantly to lower contamination rates and possibly to an increase in positivity rates especially GNB.

Conclusion

Our study demonstrates that blood volume was a variable that influenced the positivity of blood cultures using the BACTEC Aerobic Plus and the Anaerobic Plus vials in the BACTEC 9240 systems. Blood culture bottles, inoculated with > 10 ml of blood are more likely to be positive for GNB than bottles with lesser volume. These vials were also less likely to be contaminated, with a significant p value.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Bioefficacy of *Trichoderma* isolates against soil-borne pathogens

N. Srinivasa[1], T. Prameela Devi[1], S. Sudhirkumar[2]*, Deeba Kamil[1], Jyoti Lekha Borah[1] and N. Prabhakaran[1]

[1]Division of Plant Pathology, Indian Agricultural Research Institute (IARI), Pusa campus, New Delhi-110012, India.
[2]Department of Agricultural Entomology, UAS, GKVK, Bengaluru-560 065, Karnataka, India.

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The study of morphology and bioefficacy of *Trichoderma* was undertaken to select the effective isolates against soil-borne pathogens. Fifty one (51) isolates (23 isolates of *Trichoderma virens* and 28 isolates of *Trichoderma harzianum*) were morphologically characterised based on the growth characteristics on PDA medium, the size and shape of phialides and conidia.These isolates were screened for bioefficacy against soil borne plant pathogens (*Fusarium oxysporum, Rhizoctonia solani* and *Sclerotium rolfsii*) based on their percent inhibition observed during dual culture, volatile and non-volatile methods. Eight *T. virens* isolates and 12 *T. harzianum* isolates were proven to be potential isolates against the soil-borne pathogens tested. No correlation was found between bioefficacy and morphology in both species isolates.

Key words: *Trichoderma virens*, *Trichoderma harzianum*, morphology, dual culture, biocontrol and bioefficacy.

INTRODUCTION

Trichoderma spp. is cosmopolitan and abundant fungi in soil in a wide range of ecosystems and climatic zones. They are characterized by rapid growth, capability of utilizing diverse substrates and resistance to noxious chemicals (Klein and Eveleigh, 1998). Their economic importance includes their role as primary decomposers, producers of antibiotics and enzymes as well as biocontrol agents against a wide range of plant pathogens (Hjeljord and Tronsmo, 1998; Kubicek and Penttila, 1998; Rossman, 1996; Sivasithamparam and Gisalberti, 1998). *Trichoderma* spp. may inhibit the phytopathogenic fungi either by inducing resistance and plant defence reactions or by direct confrontation through mycoparasitism and antibiosis as well as competition (Howell, 1998, 2003; Papavizas, 1985; Verma et al., 2007).

In the direct interaction between *Trichoderma* spp. and the phytopathogenic fungi, mycoparasitism is one of the mechanisms observed in which the antagonist coils around the hyphae of the pathogen, develops hook-like structures known as appressoria coupled with production of lytic enzymes and then penetrates the pathogen hyphae (Chet, 1987; Kubicek et al., 2001). Coiling of the phytopathogenic fungal hyphae by *Trichoderma* spp. is one of the parameters used to characterize the mycoparasitism (Howell, 2003; Rocha-Ramirez et al., 2002). *Trichoderma* spp. produces a plethora of secondary metabolites showing anti microbial activity (Vinale et al., 2008). The chemical composition of secondary metabolites depends on the strains and classified as volatile (water-soluble) or non-volatile (water-insoluble) compounds (Ghisalberti and Sivasithamparam, 1991).

*Corresponding author. E-mail: srinivasa @ iari.res.in or cnuupath@gmail.com.

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The knowledge of mechanisms of interaction of *Trichoderma* spp. with phytopathogenic fungi and plant host is of utmost importance to enhance the practical application of these beneficial microorganisms. *Trichoderma* spp. is among the microorganisms most frequently used as antagonists against soil-borne pathogens (Hjeljord and Tronsmo, 1998 and Hyakumachi et al., 1996). Soil-borne phytopathogens are known worldwide for causing root diseases in diverse cultures (Ogoshi, 1996).

Taxonomy of *Trichoderma* is currently based largely on morphological characters such as mycelia growth, phialides shape and size and conidial shape and size. However, most species descriptions are based on examination of a limited number of strains where the morphological differences are clear but these differences become less clear as more strains are studied. This result suggests that there are no enough morphological and cultural characters to reliably define species level (Samuels et al., 2013).

The isolates were identified using morphological characters. *In vitro* bioefficacy tests (dual culture, volatile and non-volatile methods) were performed, against soil-borne pathogens to understand the ability of these isolates to produce water-soluble metabolites or volatile inhibitors. This approach is useful in selecting some potential isolates of *Trichoderma* spp. against soil-borne pathogens.

MATERIALS AND METHODS

Morphological characterisation of *Trichoderma* isolates

The cultural characteristics of 51 isolates of *Trichoderma* spp. were studied in potato dextrose agar (PDA). The identification was performed using an interactive key for strain identification (Rifai, 1969; Domsch et al., 1980; Bissett, 1991 a, b; Samuels et al., 2013) based on the growth characters on PDA along with microscopic observations of the isolates. Conidiophores branching and apex of the conidiophore disposition, shape and size of the phialides and conidia size and shape were recorded. The photographs were taken under 100x magnification (phialides size and shape and conidial size and shape) and under 10x (conidiophore branching) magnification were measured in micrometer by using ImageJ software.

Bioefficacy of *Trichoderma* isolates against soil borne pathogens

Soil-borne pathogens

Soil-borne plant pathogens (*Fusarium oxysporum, Rhizoctonia solani* and *Sclerotium rolfsii*) were obtained from Indian Type Culture Collection, Division of Plant Pathology, Indian Agricultural Research Institute (IARI) and identified based on the morphological characters (Rangaswami, 1958) and maintained in the PDA slants by repeated subculturing throughout the study.

Trichoderma isolates

Bioefficacy of *Trichoderma* isolates against three soil-borne plant pathogens, *viz., F. oxysporum, R. solani,* and *S. rolfsii* were evaluated using dual culture technique and production of volatile and non-volatile antibiotics (Dennis and Webster, 1971a, b).

Dual culture method

Trichoderma isolates were tested for their potential to antagonize *in vitro* against three soil-borne pathogens (*F. oxysporum, R. solani,* and *S. rolfsii*) using dual culture method. The test fungus and *Trichoderma* isolates were grown on PDA at 28±2°C for a week. A disc of 5 mm of the target fungus cut from periphery of the mycelium was transferred to Petri plate with PDA. *Trichoderma* was transferred aseptically to the same plate. Each plate received two discs, one of *Trichoderma* mycelium and other of the test pathogen, placed 7 cm away from each other. The plates were incubated at $28±2^0$ C and observed after eight days for growth of antagonist and test fungus, index of antagonism as percent growth inhibition of test pathogens was calculated (Morton and Stroube, 1955) (Figures 3 and 4).

Volatile method

The volatile test was carried out to observe the production of volatile inhibitors by *Trichoderma* isolates. The upper lid of PDA plates was inoculated with agar 5 mm disc of *Trichoderma* isolates and the lower lid was inoculated with soil-borne pathogens simultaneously. The two lids were taped together with adhesive tape (Dennis and Webster, 1971b) and incubated at 28±2°C for eight days. The growth of soil-borne pathogens was recorded after 72 h. In the control, soil-borne pathogens were cultured in the same way but without *Trichoderma* isolates in the bottom plate (Dennis and Webster, 1971a) (Figures 3 and 4).

Non-volatile method

The non-volatile test was carried out to find the production of water soluble inhibitors by the *Trichoderma* isolates against soil-borne pathogens (Dennis and Webster, 1971b). The isolates of *Trichoderma* culture filtrate concentration 7.5 and 15% (v/v) was inoculated in 100 ml sterile potato dextrose broth in 250 ml conical flasks. Inoculated flasks were incubated at 28±2°C for 15 days. The culture was filtered through Whatman No.42 filter papers and filtrate was collected in a sterile flask. The culture filtrate was added to molten PDA medium to obtain a final concentration of 10% (v/v). The medium was poured into the Petri plates at 15 ml/plate and 5 mm discs of pathogens were inoculated after solidification. Control plates were maintained without amending the culture filtrate. Petri plates were sealed with parafilm tape and incubated at 28±2°C for 8 days. Radial growth of soil-borne pathogens was recorded (Figures 3 and 4) and percent inhibition was calculated as per formulae adopted by Garcia (1991) as:

$$IRG (\%) = 100 [(R1-R2) / R1]$$

where R1 is the farthest radial distance grown by the pathogen in the direction of the antagonist (control) while R2 represents the distance grown on a line between inoculation positions of the pathogen and the antagonist.

Grouping of *T. virens* and *T. harzianum* isolates based on percent inhibition against soil-borne pathogens.

To select potential isolates of *Trichoderma* species effective against soil-borne pathogens, grouping has been done based on percent

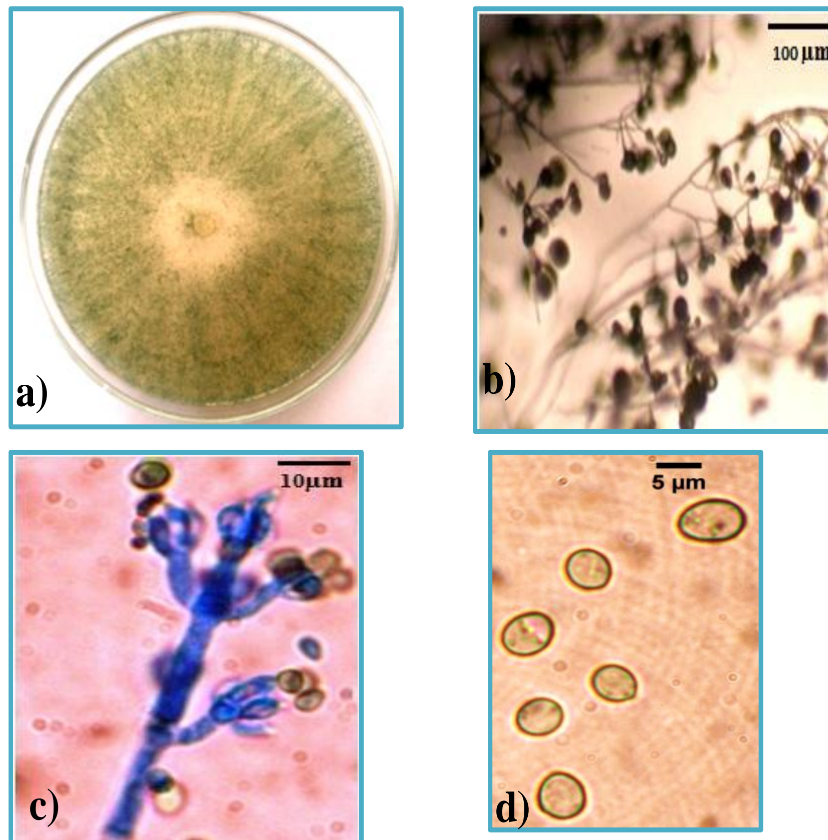

Figure 1. Morphological characters of *Trichoderma virens* isolates. a) Mycelial characters on PDA; b) Conidiophores branching in culture tube; c) Phialides; d) Conidia.

inhibition (modified Bell's scale method). Group 1: (>75-100%), Group 2: (50-75%) and Group 3: (<50%) considered as high, moderate and low potential isolates, respectively (Tables 5 and 6).

RESULTS

Morphological identification of *Trichoderma* isolates

Morphological characters such as growth characteristics, phialides disposition, shape and size and conidial shape and size of *Trichoderma* isolates were studied through microscopy and 23 isolates were confirmed as *T. virens* (dark coloured, conidiation effuse, covering the entire plate to green flat pustules concentrated near the margin and 28 isolates *T. harzianum* (pea coloured loosely aggregated flat pustules spread throughout the plate was observed in most of the isolates. Colourless to dark brown colour was observed at the reverse side of the plate) based on key given by Giddens et al. (1958), Rifai (1969) and Bissett (1984, 1991a, b) (Figures 1 and 2)

Concerning *T. virens*, the highest cultural growth was observed in the isolate V-19 (82.50 mm) and lowest in the isolateV-8 (47.50 mm). The length, width at the middle and width at the base of phialides in isolates of *T. virens* studied were from 5.80 (V-10) to 11.21(V-22) µm, 1.40 (V-20) to 3.07 (V-19) µm and 0.99 (V-20) to 2.38 (V-19) µm, respectively. Phialides shape was ampulliform in all *T. virens* isolates studied. Length and width of conidia of isolates were significantly varied ranging from 5.00 (V-1) to 6.59 (V-6) µm and 4.04 (V-1) to 5.26 (V-6) µm, respectively. The length/width (L/W) ratio of conidia ranged from 1.08 (V-22) to 1.31 (V-7) µm. Conidial shape of was obvoid to broadly ellipsoidal in all the *T. virens* isolates (Table 1).

In *T. harzianum* isolates the highest colony growth was obtained with the isolate H-10 (80 mm) and lowest with the isolate H-7 (43.50 mm). The length, width at the middle and width at the base of phialides in the isolates studied ranged from 7.87 (H-18) to 14.52 (H-22) µm and 2.25 (H-11) to 4.23 (H-25) µm and 1.54 (H-9) to 3.27 (H-5) µm, respectively. Phialides shape was ampulliform and laginiform. Length and width of conidia of isolates significantly varied from 3.50 (H-13) to 4.73 (H-17) µm and 2.76 (H-1) to 3.64 (H-20) µm, respectively. The length/width (L/W) ratio of conidia ranged from 1.12 (H-6) to 1.41 (H-5) µm. Conidial shape of *T. harzianum* was obvoid to globose (Table 2).

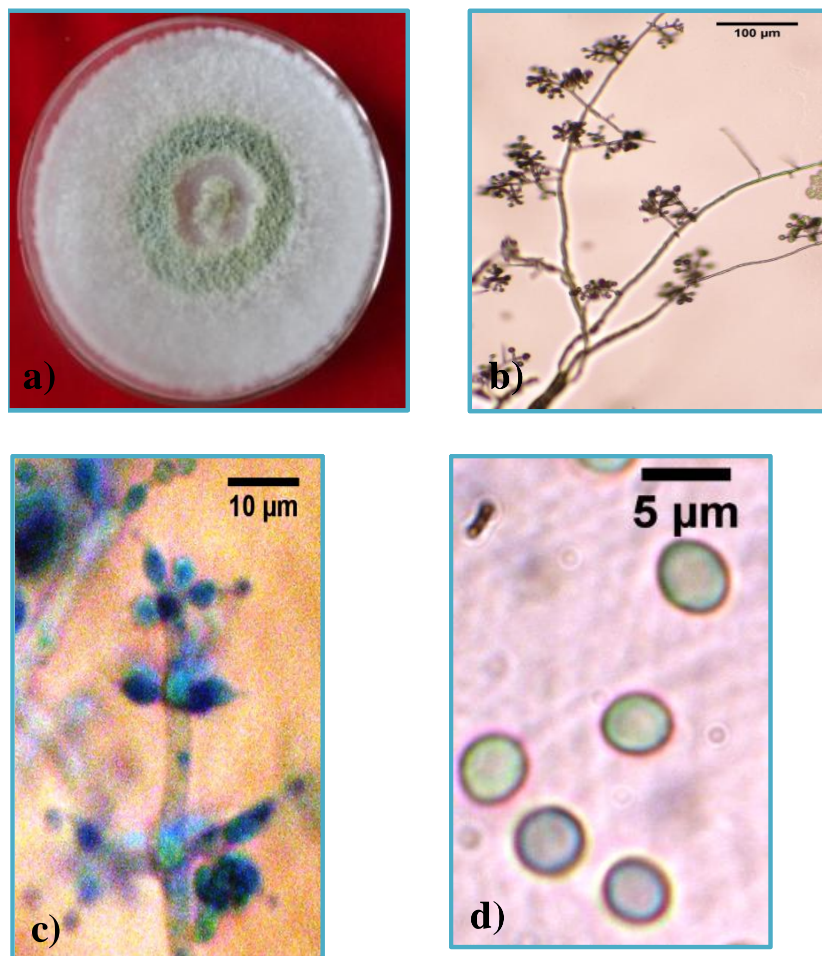

Figure 2. Morphological characters of *Trichoderma harzianum* isolates. a) Mycelial characters on PDA; b) Conidiophores branching in culture tube; c) Phialides; d) Conidia.

Bioefficacy of *Trichoderma* species isolates against soil borne pathogens

Effect of Trichoderma virens on radial growth of the soil-pathogens

Dual culture method: To select the effective bio-agents of *Trichoderma* isolates against soil-borne plant pathogens *viz., F. oxysporum, R. solani* and *S. rolfsii* (Figure 4), dual culture technique was used. All isolates of *T. virens* inhibited the mycelial growth of the soil-borne plant pathogen significantly over control. Among 23 isolates of *T. virens,* isolate V-9 inhibited the growth of *F. oxysporum* up to 82.31% which was significantly superior over all other isolates, while the isolates V-12 (53.88%) and V-5 (54.85%) showed the lowest inhibition. Isolate V-21 showed the highest percent inhibition of *R. solani* growth (81.76%) as compared to other isolates studied, while the isolate V-4 (42.93%) and V-18 (50.03%) showed the lowest inhibition. Isolate V-8 showed the highest percent inhibition of *S. rolfsii* growth (87.39%) which was significantly superior to all other isolates, while the isolates V-12 (50.23%) and V-6 (51.47%) showed the lowest inhibition (Figure 3 and Table 3).

Volatile method: Among 23 *T. virens* isolates tested for their effect of antifungal volatile metabolites production against the soil-borne plant pathogens, isolate V-7 (77.71%) was the most effective on mycelial growth of *F. oxysporum* followed by isolates V-16 (69.21%). The isolates V-4 (35.79%) and V-6 (41.05%) were the least effective against *F. oxysporum*. Isolate V-17 recorded maximum growth inhibition (76.98%) against *Rhizoctonia solani* followed by the isolates V-12 (74.84%). The isolates V-13 (52.53%) and V-15 (55.71%) were the least effective. Isolate V-8 recorded maximum growth inhibition (88.93%) against *S. rolfsii* followed by the V-22 (85.78 %). The isolates V-11 (37.82%) and V-2 (41%) were the least effective (Table 3 and Figure 3).

Table 1. Morphological characters used for the identification of *T. virens* isolates.

Strain	Culture growth on PDA (mm) at 26±2°C 3 days	Phialides				Conidia				References
		Shape	Size(µm)			Shape	Size (µm)			
			Length	Width at middle	Width at base		Length (L)	Width (W)	L/W ratio	
V-1	70.00	Ampulliform	9.714	2.078	1.504	Obvoid to broadly ellipsoidal	**5.006**	**4.044**	1.238	Rifai (1969) and Bisset (1991b)
V-2	63.50		7.159	1.843	1.259		5.312	4.559	1.165	
V-3	66.00		7.891	2.293	1.201		5.520	4.346	1.270	
V-4	69.00		8.132	2.991	1.823		5.506	4.600	1.197	
V-5	63.50		9.277	2.516	1.455		6.010	5.061	1.188	
V-6	52.50		6.439	1.716	1.170		**6.594**	**5.268**	1.252	
V-7	55.00		8.031	2.226	1.331		5.654	4.313	**1.311**	
V-8	**47.50**		9.089	2.473	1.555		5.578	4.618	1.208	
V-9	71.00		6.468	1.849	1.197		6.376	5.250	1.215	
V-10	63.00		**5.805**	1.973	1.263		6.058	4.805	1.261	
V-11	75.00		6.600	1.855	1.326		5.803	4.607	1.260	
V-12	70.00		7.800	2.234	1.475		6.064	4.880	1.243	
V-13	79.00		9.964	1.709	1.211		6.133	4.874	1.258	
V-14	75.00		8.835	2.280	1.661		6.459	5.004	1.291	
V-15	75.00		8.235	1.876	1.359		5.572	4.615	1.207	
V-16	73.00		7.184	1.974	1.352		6.173	4.751	1.299	
V-17	65.50		8.206	1.915	1.270		5.397	4.574	1.180	
V-18	66.00		6.426	1.490	1.389		5.465	4.290	1.274	
V-19	**82.50**		9.351	**3.079**	**2.380**		5.650	4.660	1.212	
V-20	61.50		7.445	**1.401**	**0.998**		5.817	5.156	1.128	
V-21	77.50		6.933	2.047	1.221		5.535	5.030	1.100	
V-22	79.00		**11.210**	2.521	2.311		5.368	4.958	**1.083**	
V-23	80.00		10.170	2.852	2.211		5.018	4.169	1.204	
S. Em±	0.17		1.07	0.06	0.07		0.19	0.10	0.01	
CD (p=0.05)	1.67		4.22	1.00	1.13		1.03	0.75	0.27	

Values in bold indicate highest and lowest sizes and culture growth of *T. virens* isolate.

Non-volatile method: Crude antibiotics produced from culture filtrate of the *T. virens* isolates were tested on radial growth of the soil-borne pathogens. All the isolates were found to reduce the radial growth of the pathogens over the control. Isolate V-23 (81.80%) recorded the maximum growth inhibition of *F. oxysporum* growth followed by the isolate V-19 (80.15%). The isolates V-3 (58.92%) and V-16 (59.01%) were the least effective. Isolate V-19 (71.61%) showed maximum growth inhibition of *R. solani* followed by the isolates V-23 (70.14%). The isolates V-18 (43.32%) and V- 5 (47.77%) were the least effective. Maximum growth inhibition (83.22%) was observed with *S. rolfsii* by the isolate V-9

Table 2. Morphological characters used for the identification of *T. harzianum* isolates.

Strain	Culture growth on PDA (mm) at 26±2°C 3 days	Phialides				Conidia				References
		Shape	Size(µm)			Shape	Size(µm)			
			Length	Width at middle	Width at base		Length (L)	Width (W)	L/W ratio	
H-1	50.00	Lagini form and ampulliform	10.896	3.496	2.435	Obvoid to Globose	3.561	**2.760**	1.290	Bisset (1991b)
H-2	64.50		11.609	3.507	2.081		3.759	3.025	1.242	
H-3	61.00		10.883	4.281	3.182		3.855	3.015	1.279	
H-4	47.50		10.549	3.136	1.965		4.220	3.169	1.332	
H-5	51.00		11.918	4.231	**3.275**		4.289	3.032	**1.415**	
H-6	51.00		12.113	2.761	2.102		3.590	3.180	**1.129**	
H-7	**43.50**		12.808	3.249	2.112		3.772	2.847	1.325	
H-8	45.00		12.804	2.458	1.786		3.720	3.232	1.151	
H-9	53.50		9.958	2.327	**1.546**		4.006	2.913	1.375	
H-10	**80.00**		13.858	3.074	1.911		4.475	3.185	1.405	
H-11	52.00		9.219	**2.259**	1.711		4.050	3.118	1.299	
H-12	48.00		11.663	3.387	2.053		4.394	3.182	1.381	
H-13	50.00		13.282	3.817	2.558		**3.502**	3.079	1.138	
H-14	66.50		14.504	3.786	2.719		4.364	3.279	1.331	
H-15	47.50		12.013	3.585	2.541		4.575	3.418	1.338	
H-16	70.00		9.756	3.719	2.533		4.058	3.212	1.263	
H-17	50.00		10.682	2.859	2.588		**4.735**	3.531	1.341	
H-18	50.50		**7.878**	3.621	2.223		4.079	3.440	1.186	
H-19	48.00		12.247	3.528	2.143		4.112	3.379	1.217	
H-20	51.00		11.953	3.438	2.094		4.152	**3.640**	1.141	
H-21	57.00		9.929	3.099	1.786		4.115	3.346	1.230	
H-22	49.00		**14.523**	3.198	3.141		3.880	3.398	1.142	
H-23	51.00		10.678	2.876	2.584		4.328	3.291	1.315	
H-24	53.00		10.839	3.194	2.161		3.885	3.330	1.167	
H-25	49.50		10.199	**4.238**	2.896		3.910	3.210	1.218	
H-26	61.00		12.078	2.743	2.592		3.649	3.032	1.203	
H-27	47.50		12.159	2.733	2.391		3.990	3.397	1.174	
H-28	60.00		13.806	3.331	2.348		4.706	3.335	1.411	
S.Em±	0.06		3.21	0.06	0.10		0.08	0.09	0.02	
C.D (p=0.05)	1.06		7.37	1.08	1.33		0.68	0.73	0.34	

Values in bold indicate highest and lowest sizes and culture growth of *T. harzianum* isolate.

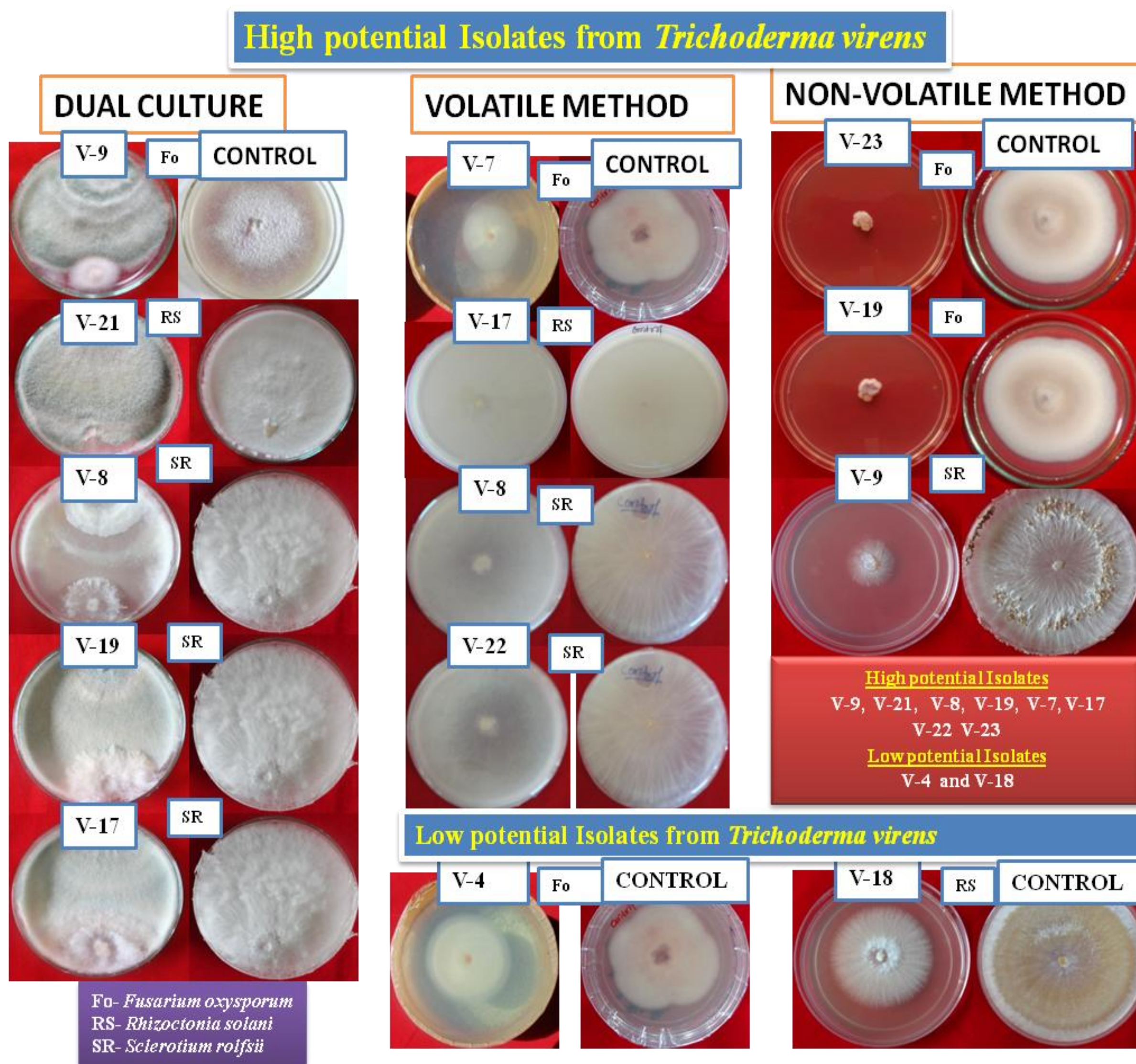

Figure 3. List of high and low potential isolates of *Trichoderma virens* selected from the bioefficacy methods.

followed by the isolate V-8 (78.36%). The isolates V-21 (51.35%) and V-18 (54.50%) were the least effective (Table 3 and Figure 3).

Among all the isolates of *T. virens* tested against three different soil-borne pathogens, highest percent mean inhibition was observed by the isolates V-9 (76.51%) and V-19 (72.38%) with *F. oxysporum.* Isolates V-19 (68.01%) and V-12 (67.37%) with *R. Solani* and isolate V-8 (84.89%) with *S. rolfsii* (Table 7).

Effects of Trichoderma harzianum on radial growth of the soil-pathogens

Dual culture method: Similarly, among 28 isolates of *T. harzianum* tested, isolate H-12 inhibited the growth of *F. oxysporum* upto 86.23% which was significantly superior over all other isolates, followed by isolates H-3 (81.35%), while the isolates H-9 (54.92%) and H-24 (60.77%) showed lowest inhibition. Isolate H-26 (84.58%) showed highest percent inhibition of *R. solani* growths as compared to other isolates studied, followed by isolates H-16 (84.50%). The isolates H-6 (55.46%) and H-15 (56.17%) showed the lowest inhibition. Isolate H-11 (85.75%) showed highest percent inhibition of *S. rolfsii* which was significantly superior to all other isolates followed by H-18 (84.42%), while the isolate H-16 (61.25%) and H-14 (64.58%) showed the lowest inhibition (Table 4 and Figure 4).

Volatile method: Isolate H-3 (83.77 %) was found to be

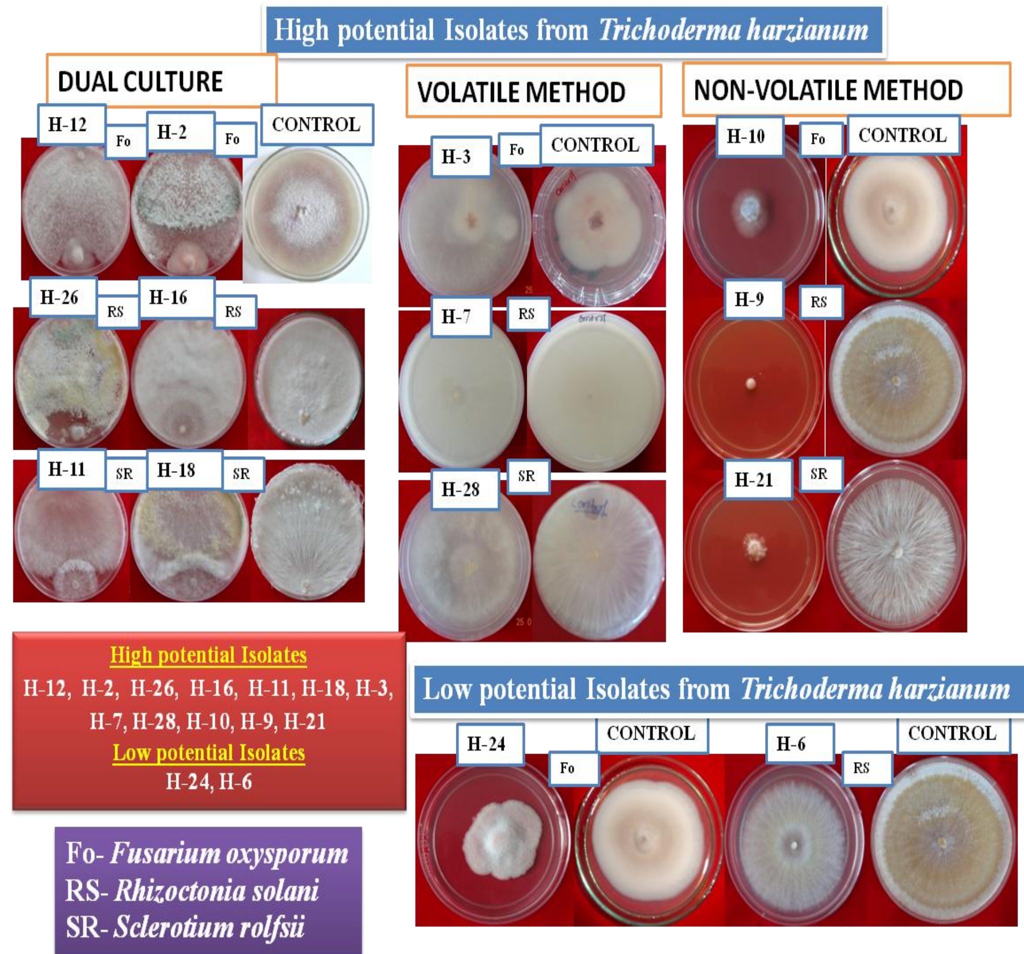

Figure 4. List of high and low potential isolates of *Trichoderma harzianum* selected from the bioefficacy methods.

more effective against *F. oxysporum* pathogen followed by the isolate H-26 (82.47%). The isolates H-6 (65.04%) and H-1 (66.29%) were the least effective. Isolate H-7 (82.63%) recorded maximum growth inhibition in *R. solani* followed by the isolates H-15 (79.48 %). The isolates H-2 (57.42%) and H-9 (59.00%) were least effective. Isolate H-28 (76.65%) recorded maximum growth inhibition against *S. rolfsii* followed by H-2 (76.59%) and the isolates H-5 (50.61%) and H-1, H-5 (50.61%) were least effective (Table 4 and Figure 4).

Non-volatile method: Isolate H-10 recorded maximum growth inhibition (81.89 %) in *F. oxysporum* followed by the isolates H-16 (78.04%) and the isolates H-24 (39.74%) and H-23 (51.92%) were least effective. Isolate H-9 (87.19%) recorded maximum growth inhibition against *R. solani* followed by the isolate H-7 (83.42%) H-6 (25.53%) and H- 24 (56.45%) were least effective. Isolate H-21 (86.04%) recorded maximum growth inhibition in *S. rolfsii* followed by the isolates H-23 (83.66%) and the isolates H-7 (63.91%) and H-8 (65.10%) are least effective (Table 4 and Figure 4).

Isolate H-3 (77.70%) showed highest percent mean inhibition of *F. oxysporum,* H-18 (76.29%) in *R. solani* and H-2 (76.35%) in *S. rolfsii* (Table 7).

Results (Tables 5 and 6) of mycelial growth percent inhibition for screening of *Trichoderma* isolates against soil-borne pathogens revealed that there was a clear difference within the isolates of *T. virens* and *T. harzianum* with respect to their percent inhibition against the pathogens tested in different methods used. It appeared that large numbers of *T. virens* and *T. harzianum* isolates fell under the category of Group 2 and very few numbers fell under category of Group 1. From the grouping, eight high potential (V-7, V-8, V-9, V-17, V-19, V-21, V-22 and V-23) and two low potential (V-4 and V-18) isolates from *T. virens* and twelve high potential (H-2, H-3, H-7, H-9, H-10, H-11, H-12, H-16, H-18, H-21, H-26 and H-28) and 2 low potential (H-6 and H-24) isolates

Table 3. Effect of *Trichoderma virens* on radial growth of soil-borne pathogens on PDA at 26± 2°C at 8 DAI.

Isolate	Percent inhibition of *Fusarium oxysporum* growth				Percent inhibition of *Rhizoctonia solani* growth				Percent inhibition of *Sclerotium rolfsii* growth				Dual Mean	Volatile Mean	Non-volatile Mean	Grand Mean
	Dual	Volatile	Non-volatile	Mean	Dual	Volatile	Non-volatile	Mean	Dual	Volatile	Non-volatile	Mean				
V-1	68.62	47.43	72.61	62.89	68.33	58.57	55.21	60.70	68.04	53.57	63.60	61.74	68.33	53.19	63.81	61.78
V-2	71.65	43.42	69.67	61.58	64.77	63.08	61.19	63.01	72.55	41.00	63.80	59.12	69.66	49.17	64.89	61.24
V-3	76.46	66.71	**58.92**	67.36	61.72	59.34	56.73	59.26	62.29	54.34	69.56	62.06	66.82	60.13	61.74	62.90
V-4	66.69	**35.79**	66.54	**56.34**	**42.93**	61.54	54.43	**52.97**	73.07	46.50	68.65	62.74	60.90	47.94	63.21	57.35
V-5	54.85	65.46	75.55	65.29	60.60	66.76	47.77	58.38	54.25	62.70	71.78	62.91	56.57	64.97	65.03	62.19
V-6	68.69	41.05	72.79	60.84	73.56	70.22	49.96	64.58	51.47	60.79	73.51	61.92	64.57	57.35	65.42	62.45
V-7	64.62	**77.71**	69.85	70.73	59.40	59.34	65.64	61.46	55.36	65.04	69.15	63.18	59.79	67.36	68.21	65.12
V-8	80.38	57.76	62.13	66.76	67.61	57.14	56.68	60.48	**87.39**	**88.93**	78.36	**84.89**	78.46	67.94	65.72	70.71
V-9	**82.31**	68.55	78.68	**76.51**	79.37	60.77	53.74	64.63	55.36	80.40	**83.22**	72.99	72.35	69.91	71.88	71.38
V-10	74.54	46.05	69.49	63.36	61.79	58.57	55.17	58.51	74.90	47.27	64.91	62.36	70.41	50.63	63.19	61.41
V-11	65.77	56.45	75.55	65.92	73.56	70.33	58.16	67.35	59.97	**37.82**	71.78	56.52	66.43	54.87	68.50	63.27
V-12	**53.88**	55.07	75.74	61.56	70.58	74.84	56.68	67.37	**50.23**	73.18	75.73	66.38	58.23	67.70	69.38	65.10
V-13	68.65	57.76	69.85	65.42	75.30	**52.53**	55.21	61.01	73.07	41.74	72.69	62.50	72.34	50.68	65.92	62.98
V-14	63.73	67.00	69.67	66.80	55.26	64.51	56.73	58.83	76.01	54.34	56.20	62.18	65.00	61.95	60.87	62.61
V-15	69.58	51.32	60.66	60.52	52.93	55.71	58.20	55.61	68.63	71.56	68.65	69.61	63.71	59.53	62.50	61.92
V-16	72.62	69.21	59.01	66.95	60.56	61.65	53.74	58.65	64.58	42.54	64.71	57.28	65.92	57.80	59.15	60.96
V-17	61.85	53.95	63.60	59.80	55.84	**76.98**	65.48	66.10	82.94	71.71	71.29	75.31	66.88	67.55	66.79	67.07
V-18	68.62	57.76	74.17	66.85	50.03	67.25	**43.32**	53.53	54.31	42.51	54.50	**50.44**	57.65	55.84	57.33	56.94
V-19	71.54	65.46	80.15	**72.38**	63.51	68.90	**71.61**	**68.01**	83.40	64.85	64.21	70.82	72.82	66.40	71.99	70.40
V-20	72.62	56.45	69.67	66.25	51.77	57.91	58.24	55.97	53.07	53.54	55.91	54.17	59.15	55.97	61.27	58.80
V-21	76.54	64.08	69.49	70.04	**81.76**	61.65	57.44	66.95	64.51	64.45	**51.35**	60.10	74.27	63.39	59.43	65.70
V-22	78.62	65.46	63.60	69.23	63.51	60.11	62.66	62.09	78.24	85.78	68.65	77.56	73.46	70.45	64.97	69.63
V-23	56.85	66.71	**81.80**	68.45	54.13	58.57	70.14	60.95	73.07	70.10	58.13	67.10	61.35	65.13	70.02	65.50
Mean	69.12	58.11	69.96	65.73	62.99	62.88	57.57	61.15	66.81	59.77	66.97	64.52	66.31	60.25	64.84	63.80
SEm±	21.66	11.32	32.60		15.52	14.55	6.87		5.46	11.30	44.48					
CD (p=0.5)	18.93	13.68	23.22		16.02	15.51	10.66		9.50	13.68	27.12					

Values in bold indicate highest and lowest sizes for the different soil-borne pathogens.

from *T. harzianum* were selected as a promising isolates for the further studies. The high and low potential isolates showed highest and lowest percent inhibition respectively in the three methods used (Table 7).

DISCUSSION

The present findings suggested that the broad conidiophore, terminated by a cluster of 3-6 closely adpressed phialides, whorls of 2-5, ampulliform phialides, dull blackish green color mycelium, effuse conidiation, broadly ellipsoid to obvoid, conidia in case of *T. virens*. Similarly in case of *T. harzianum*, whorls of phialides 2-6, ampulliform to laginiform, narrower at the base,

Table 4. Effect of *Trichoderma harzianum* on radial growth of soil-borne pathogens on PDA at 26± 2°C at 8 DAI.

Isolate	Percent inhibition of *Fusarium oxysporum* growth				Percent inhibition of *Rhizoctonia solani* growth				Percent inhibition of *Sclerotium rolfsii* growth				Dual Mean	Volatile Mean	Non-volatile Mean	Grand Mean
	Dual	Volatile	Non-volatile	Mean	Dual	Volatile	Non-volatile	Mean	Dual	Volatile	Non-volatile	Mean				
H-1	74.46	66.29	55.77	65.51	70.33	72.44	69.21	70.66	72.83	50.61	70.94	64.79	72.54	63.11	65.31	66.99
H-2	80.46	72.08	59.94	70.82	57.42	**57.42**	74.34	63.06	78.00	76.59	74.46	**76.35**	71.96	68.70	69.58	70.08
H-3	81.35	**83.77**	67.98	**77.70**	72.83	77.94	62.76	71.18	76.75	71.46	79.11	75.77	76.98	77.72	69.95	74.88
H-4	74.54	79.11	58.17	70.61	65.17	70.02	62.83	66.01	79.42	62.31	68.61	70.12	73.04	70.48	63.20	68.91
H-5	78.38	67.42	63.78	69.86	67.04	73.18	61.51	67.24	74.83	50.61	72.19	65.87	73.42	63.74	65.83	67.66
H-6	74.54	**65.04**	63.78	67.79	**55.46**	65.26	**25.53**	**48.75**	75.50	66.19	66.23	69.31	68.50	65.50	51.85	61.95
H-7	70.54	76.62	63.91	70.36	61.25	**82.63**	83.42	75.77	82.00	62.31	**63.91**	69.41	71.26	73.85	70.41	71.84
H-8	79.46	69.70	70.19	73.12	71.50	62.11	74.47	69.36	72.00	59.75	65.10	65.92	74.63	63.85	69.92	69.47
H-9	**54.92**	76.73	65.87	65.84	66.50	59.00	**87.17**	70.89	72.92	68.83	74.46	71.76	64.47	68.19	75.83	69.50
H-10	76.46	69.75	**81.89**	76.03	76.67	63.80	71.84	70.77	74.83	68.89	70.89	71.54	75.99	67.48	74.87	72.78
H-11	74.46	74.46	52.24	67.05	70.17	73.95	69.21	71.11	**85.75**	62.31	69.70	72.59	76.79	70.24	63.72	70.25
H-12	**86.23**	79.00	56.09	73.77	82.08	65.33	62.83	70.08	78.08	66.19	74.51	72.93	82.13	70.17	64.48	72.26
H-13	70.58	72.08	63.94	68.87	66.33	73.18	66.71	68.74	75.50	64.91	72.08	70.83	70.80	70.06	67.58	69.48
H-14	69.00	69.81	75.96	71.59	62.58	76.33	65.46	68.12	64.58	59.75	69.81	64.71	65.39	68.63	70.41	68.14
H-15	64.62	69.70	68.11	67.47	56.17	79.48	64.08	66.57	78.71	63.63	74.46	72.27	66.50	70.94	68.88	68.77
H-16	72.54	80.19	78.04	76.93	84.50	73.25	56.45	71.40	**61.25**	59.75	67.42	**62.81**	72.76	71.06	67.30	70.38
H-17	66.69	69.70	69.87	68.75	59.92	66.95	74.47	67.11	78.08	63.60	74.35	72.01	68.23	66.75	72.90	69.29
H-18	80.38	81.49	55.77	72.55	74.17	77.87	76.84	**76.29**	84.42	73.95	69.75	76.04	79.66	77.77	67.45	74.96
H-19	62.62	74.46	54.33	63.80	65.08	65.41	69.21	66.57	81.92	64.88	75.49	74.10	69.87	68.25	66.34	68.15
H-20	70.58	74.35	48.08	64.33	61.25	68.41	60.26	63.31	78.00	53.17	70.89	67.35	69.94	65.31	59.74	65.00
H-21	78.46	79.00	71.79	76.42	62.50	76.18	81.98	73.55	74.17	61.00	**86.04**	73.73	71.71	72.06	79.94	74.57
H-22	66.69	69.81	52.24	62.91	71.58	66.14	71.84	69.86	71.58	55.80	79.00	68.80	69.95	63.92	67.69	67.19
H-23	68.62	69.70	51.92	63.41	66.42	65.26	66.58	66.09	74.17	**50.61**	83.66	69.48	69.74	61.86	67.39	66.32
H-24	60.77	71.05	**39.74**	**57.19**	65.08	68.49	56.45	63.34	72.38	61.00	69.75	67.71	66.08	66.85	55.31	62.74
H-25	78.38	72.08	65.87	72.11	62.58	65.33	66.71	64.88	72.83	64.88	74.46	70.72	71.26	67.43	69.01	69.24
H-26	70.62	82.47	59.94	71.01	**84.58**	73.03	66.58	74.73	66.42	71.39	72.08	69.96	73.87	75.63	66.20	71.90
H-27	78.46	76.73	56.09	70.43	63.83	66.80	60.20	63.61	81.29	64.88	73.32	73.16	74.53	69.47	63.20	69.07
H-28	78.38	69.70	56.09	68.06	74.08	73.18	66.71	71.32	78.75	**76.65**	72.08	75.83	77.07	73.18	64.96	71.74
Mean	72.97	73.65	61.69	69.44	67.75	69.94	66.99	68.23	75.61	63.43	72.67	70.57	72.11	69.01	67.12	69.41
SEm±	9.28	12.86	29.83		10.08	22.23	10.61		4.99	19.64	9.05					
CD(p=0.05)	12.51	14.73	22.44		13.05	19.37	13.39		9.18	18.21	12.36					

Values in bold indicate highest and lowest sizes for the different soil-borne pathogens.

whitish green to pale green color mycelium, conidiation effuse, covering the entire surface of the plate, globose to sub-globose conidia. In this study, colony morphology, phialides and conidial morphology and size could separate *Trichoderma* spp. into *T. virens* and *T. harzianum*. These findings are duly supported by earlier observations (Rifai, 1969; Domsch et al., 1980, Bissett, 1991a, b; Samuel, 1996, 2006) where they

Table 5. Grouping of *Trichoderma virens* isolates based on percent inhibition against soil-borne pathogens.

Inhibition	Group*	*Fusarium oxysporum*			*Rhizoctonia solani*			*Sclerotium rolfsii*		
		Dual culture	Volatile	Non-Volatile	Dual culture	Volatile	Non-Volatile	Dual culture	Volatile	Non-Volatile
High	Group-1 (75-100% Inhibition)	V-3, V-9, V-8, V-22	V-7	V-5, V-9, V-11, V-12, V-19, V-23	V-13, V-21	V-17	0	V-8, V-14, V-19, V-17, V-22	V-8, V-22	V-8, V-9
	Number of isolates	4	1	6	2	1	0	4	2	2
Moderate	Group-2 (50-75% Inhibition)	V-1, V-2, V-4, V-5, V-6, V-7, V-8, V-10, V-11, V-12, V-13, V-14, V-15, V-16, V-17, V-19, V-20, V-22, V-23	V-3, V-5, V-8, V-9, V-11, V-12, V-13, V-14, V-15, V-16, V-17, V-18, V-19, V-20, V-21, V-22, V-23	V-1, V-2, V-3, V-4, V-6, V-7, V-8, V-10, V-13, V-14, V-15, V-16, V-17, V-18, V-20, V-21, V-22	V-1, V-2, V-3, V-5, V-6, V-7, V-8, V-9, V-10, V-11, V-12, V-14, V-15, V-16, V-17, V-18, V-19, V-20, V-22, V-23	V-1, V-2, V-3, V-4, V-5, V-6, V-7, V-8, V-9, V-10, V-11, V-12, V-13, V-14, V-15, V-16, V-18, V-19, V-20, V-21, V-22, V-23	V-1, V-2, V-3, V-4, V-7, V-8, V-9, V-10, V-11, V-12, V-13, V-14, V-15, V-16, V-17, V-19, V-20, V-21, V-22, V-23	V-1, V-2, V-3, V-4, V-5, V-6, V-7, V-9, V-10, V-11, V-12, V-13, V-15, V-16, V-17, V-18, V-20, V-21, V-23	V-1, V-3, V-5, V-6, V-7, V-9, V-12, V-14, V-15, V-17, V-19, V-20, V-21, V-23	V-1, V-2, V-3, V-4, V-5, V-6, V-7, V-10, V-11, V-12, V-13, V-14, V-15, V-16, V-18, V-17, V-19, V-20, V-21, V-22, V-23
	Number of isolates	19	18	17	20	22	20	19	14	21
Low	Group-3 (< 50 % Inhibition)	0	V-1, V-2, V-4, V-6, and V-10	0	V-4	0	V-5, V-6, V-18	0	V-2, V-4, V-10, V-11, V-13, V-16, V-18,	0
	Number of isolates	0	5	0	1	0	3	0	7	0

characterized different species of *Trichoderma*. The few morphological characters available are variable to some degree with respect to variable climatic and geographic locations, leading to overlap among species.

In the present finding, eight high potential and two low potential isolates from *T. virens* and 12 high potential and 2 low potential isolates from *T. harzianum* were selected as a promising isolates. The high and low potential isolates showed highest and lowest percent inhibition in the three methods used and against the three soil-borne pathogens tested. The possible explanation of this result may be due to their inherent potentiality to adapt well in introduced conditions (Papavizas, 1985; Bae and Knudsen, 2005), though it rarely occurs (Whipps, 2001). Higher growth rate ability of the selected strains are indicative of their better antagonistic potential. Mathur and Sarbhoy (1978) reported that *T. viride* and *T. harzianum* inhibited the growth of *S. rolfsii* by 88 and 86%, respectively. Mathew and Gupta (1998) showed that *T. harzianum* exhibited maximum antagonistic activity causing 58.3% inhibition of *F. oxysporum. f.* sp. *lycopersici, R. solani* and *S. rolfsii* followed by *T. hamatum*, *T. viride* and *T. virens* inhibition by 48.3, 46.1 and 44.9%, respectively. Recently, Noveriza and Quimio (2004) reported that *Trichoderma* spp. were able to cause 66.36% growth inhibition of *F. oxysporum. f.* sp. *lycopersici*, *R. solani* and *S. rolfsii* through dual culture technique and were also significantly inhibited by *Trichoderma* spp. *in vitro* (Lozoya-

Table 6. Grouping of *Trichoderma harzianum* isolates based on percent inhibition against soil-borne pathogens.

Inhibition	Groups*	*Fusarium oxysporum*			*Rhizoctonia solani*			*Sclerotium rolfsii*		
		Dual culture	Volatile	Non-Volatile	Dual culture	Volatile	Non-Volatile	Dual culture	Volatile	Non-Volatile
High	Group-1 (75-100% Inhibition)	H-2, H-3, H-4, H-5, H-8, H-10, H-12, H-18, H-21, H-25, H-27	H-3, H-4, H-7, H-9, H-12, H-16, H-18, H-21, H-26, H-27	H-10, H-14, H-16	H-12, H-16, H-26	H-3, H-7, H-14, H-15, H-18, H-21	H-7, H-9, H-18,	H-2, H-3, H-4, H-6, H-7, H-11, H-12, H-13, H-15, H-17, H-18, H-19, H-20, H-27, H-28	H-2, H-28	H-3, H-19, H-21, H-22, H-23
	Number of isolates	11	10	3	3	6	3	15	2	5
Moderate	Group-2 (50-75% Inhibition)	H-1, H-6, H-7, H-9, H-11, H-13, H-14, H-15, H-16, H-17, H-19, H-20, H-22, H-23, H-24, H-26, H-28	H-1, H-2, H-5, H-6, H-8, H-10, H-11, H-13, H-14, H-15, H-17, H-19, H-20, H-22, H-23, H-24, H-25, H-28	H-1, H-2, H-3, H-4, H-5, H-6, H-7, H-8, H-9, H-10, H -12, H-13, H-15, H-17, H-18, H-19, H-21, H-22, H-23, H-25, H-26, H-27, H-28	H-1, H-2, H-3 , H-4, H-5, H-6, H-7, H-8, H-9, H-10, H-11, H-13, H-14, H-15, H-17, H-18, H-19, H-20, H-21, H-22, H-23, H-24, H-25, H-27, H-28	H-1, H-2, H-4, H-5, H-6, H-8, H-9, H-10, H-11, H-12, H-13, H-16, H-17, H-19, H-20, H-22, H-23, H-24, H-25, H-26, H-27, H-28	H-1, H-2, H-3, H-4, H-5, H-8, H-10, H-11, H-12, H-13, H-14, H-15, H-16, H-17, H-19, H-20, H-21, H-22, H-23, H-24, H-25, H-26, H-27, H-28	H-1, H-5,H-8, H-10, H-9, H-14, H-16, H-21, H-22, H-23, H-24, H-25, H-26	H-1, H-3, H-4, H-5, H-6, H-7, H-8, H-9, H-10, H-11, H-12, H-13, H-14, H-15, H-16, H-17, H-18, H-19, H-20, H-21, H-22, H-23, H-24, H-25, H-26, H-27	H-1, H-2, H-4, H-5, H-6, H-7, H-8, H-9, H-10, H-11, H-12, H-13, H-14, H-15, H-16, H-17, H-18, H-20, H-24, H-25, H-26, H-27, H-28
	Number of isolates	17	18	23	25	22	24	13	26	23
Low	Group-3 (< 50 % Inhibition)	0	0	H-20, H-24	0	0	H-6	0	0	0
	Number of isolates	0	0	2	0	0	1	0	0	0

Saldana et al., 2006; Choudhary et al., 2007; Kumar and Hooda, 2007; Pan and Bhagat, 2007, 2008).

Conclusion

The objective of the present study was to investigate morphology and identification of *Trichoderma* spp. before conducting bioefficacy test *in vitro* as well as *in vivo*. Bioefficacy helps to select some promising isolates of *Trichoderma* species against soil-borne plant pathogens. Fifty one *Trichoderma* isolates obtained from Indian Type Culture Collection were morphologically characterised and identified as *T. virens* (23 isolates) and *T. harzianum* (28 isolates) on the basis of the literature reported. These isolates were tested for their bioefficacy using 3 methods (dual culture, volatile and non-volatile) against soil-borne pathogens *viz., F. oxysporum, R. solani* and *S. rolfsii.* Out of 51 isolates, 8 isolates of *T. virens* and 12 isolates of *T. harzianum* were proved as potential biocontrol agents.

Table 7. List of high and low potential isolates of *Trichoderma virens* and *T. harzianum* selected for molecular and biochemical characterization.

Name of the species	High potential	Low potential	Total	
Trichoderma virens	V-7, V-8, V-9, V-17, V-19, V-21, V-22, V-23 (8 Isolates)	V-4, V-18 (2 Isolates)	10 Isolates	24 Isolates
Trichoderma harzianum	H-2, H-3, H-7, H-9, H-10, H-11, H-12, H-16, H-18, H-21, H-26, H-28 (12 Isolates)	H-6, H-24 (2 Isolates)	14 Isolates	

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Influence of white-rot fungi on chemical composition and *in vitro* digestibility of lignocellulosic agro-industrial residues

Cláudia Braga Pereira Bento[1], Juliana Soares da Silva[1], Marcelo Teixeira Rodrigues[2], Maria Catarina Megumi Kasuya[1] and Hilário Cuquetto Mantovani[1]*

[1]Departamento de Microbiologia, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil.
[2]Departamento de Zootecnia, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

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In this work, we tested the ability of white-rot fungi to alter the chemical composition of lignocellulosic agro-industrial residues so that they could be more susceptible to microbial degradation by rumen microorganisms, thus improving *in vitro* digestibility of neutral detergent fiber. Agro-industrial residues that were incubated for 60 days with *Pleurotus ostreatus* PLO06 or *Lentinula edodes* UFV73 showed significant changes in chemical composition, increasing (P<0.05) the crude protein content and the *in vitro* digestibility of neutral detergent fiber. Lignin content and structural carbohydrates decreased after fungal fructification in most substrates and an increase (P<0.05) in dry matter mineralization was also observed for residues treated with *P. ostreatus* PLO06. *L. edodes* UFV73 required a more balanced carbon/nitrogen ratio to grow on lignocellulosic substrates, and *P. ostreatus* PLO06 was in general, more effective than *L. edodes* UFV73 to alter the chemical composition and digestibility of the agro-industrial residues. Among the residues tested, eucalyptus bark showed the highest increase in digestibility after fungal growth. The marked increments in fiber digestibility obtained with substrates inoculated with either *P. ostreatus* PLO06 or *L. edodes* UFV73 indicate the usefulness of biological pretreatments to improve the nutritive value of low-quality lignocellulosic feedstuffs that could be incorporated into ruminant rations.

Key words: *Pleurotus ostreatus*, *Lentinula edodes*, biological pretreatment, rumen, fungal fructification, lignin.

INTRODUCTION

Lignocellulosic materials are the most abundant agricultural residues in the world (Shrivastava et al., 2011) and agricultural commodities play a major role in the gross domestic product of several economies worldwide (Tuyen et al., 2013). Commercial crop production and many industrial activities can generate

*Corresponding author. E-mail: hcm6@ufv.br. Tel: + 55 (31) 3899-1946. Fax: + 55 (31) 3899-2573.

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large amounts of residues with a complex chemical composition, in which cellulose, hemicellulose and lignin are the main constituents (Dashtban et al., 2009; Sánchez, 2009).

The disposal of lignocellulosic residues has often been associated with environmental pollution as well as sanitary and ecological effects (Dashtban et al., 2009), but several lignocellulosic residues could be useful to obtain products of great economic, energetic or nutritional value, such as chemicals, biofuels and animal feeds (Okano et al., 2006; Hasunuma et al., 2012).

Ruminant animals in particular have evolved a symbiotic relationship with biomass-degrading microbes that allows them to digest lignocellulosic biomass and at least some agricultural and industrial lignocellulosic residues are used as ingredients of cattle diets (Okano et al., 2006; Sánchez, 2010; Shrivastava et al., 2011). Nonetheless, the extension of digestion and nutritional value of these residues may be limited by their content of lignin and protein. Therefore, strategies for pretreatment of lignocellulosic biomass can improve the overall digestion and the nutritional value of agricultural and industrial residues that could be used in ruminant rations (Okano et al., 2006; Fazaeli, 2007).

The biological breakdown of lignocellulosic materials depends on the hydrolytic activity of enzymes produced by a variety of microbial species, especially bacteria and fungi (Sharma and Arora, 2010; Dong et al., 2013). White-rot fungi are well known for their ability to produce hydrolytic and oxidative extracellular enzymes and decompose lignocellulosic substrates (Philippoussis et al., 2011). In this regard, the genera *Pleurotus* and *Lentinula* belong to a group of widely distributed edible mushrooms with the ability to degrade several lingocellulosic substrates (Sánchez, 2010; Philippoussis et al., 2011).

P. ostreatus is a highly ligninolytic fungus with great potential for biotechnological applications due to its simple growth requirements and ability to secrete different oxidative enzymes, such as peroxidases and laccases, active against a variety of toxic substances and recalcitrant substrates (Sánchez, 2010; Shrivastava et al., 2011). Previous work demonstrated strain-specific variations in mushroom production and *P. ostreatus* PLO06 showed high activities of manganese peroxidase, xylanase and cellulase growing in a variety of complex and growth conditions (Luz et al., 2012).

The basidiomycete *Lentinula edodes* (Berkeley) Pegler (*Lentinus edodes*), known as shiitake, is the second most popular mushroom in the world (Rincão et al., 2012). *L. edodes* is valued for its nutritional and medicinal properties, culinary and industrial applications (Jiang et al., 2010) as well as biodegradation and biotransformation abilities (Lakhtar et al., 2010). Strains of *L. edodes* produce extracellular oxidizing enzymes capable of biodegrading lignin-related recalcitrant compounds, such as polyphenols.

P. ostreatus and *L. edodes* have been studied as an alternative to improve the nutritional value of lignincelulosic substrates as ruminant feeds (Tuyen et al., 2012; Dong et al., 2013; Tuyen et al., 2013). However, the majority of these previous studies focused only on substrates with a very limited range of neutral detergent fiber digestibility and lignin concentrations (Okano et al., 2006, Lynch et al., 2013). Furthermore, no previous study has been conducted to address the simultaneous use of lignocellulosic substrates for mushroom production and animal feeds. This technology could be profitable especially in small farms, where the use of abundant agro-industrial substrates could serve as a source of income to farmers who could commercialize the mushrooms harvested from fructified substrates and use these as substrates with greater nutritional value in ruminant feeds.

In the present study, we explored the ability of *P. ostreatus* PLO06 and *L.edodes* UFV73 to degrade lignin and other recalcitrant substrates at a high rate to: 1) determine their ability to improve the protein content of several agro-industrial residues with different chemical compositions and concentrations of digestible carbohydrates and 2) improve the *in vitro* digestibility of different lignocellulosic materials that could also be applied for mushroom production.

Although some substrates used in this study usually have low nutritional quality to be included directly into livestock diets, we hypothesized that the fungal biomass could improve the nutritional value of these residues by increasing the concentration of protein and digestibility of the substrates as well as reducing the lignin content of the lignocellulosic residues.

MATERIALS AND METHODS

Microorganism and growth conditions

P. ostreatus PLO06 and *L.edodes* UFV73 were isolated from eucalyptus sawdust and were obtained from the culture collection maintained at the Mycorrhizal Associations Laboratory/BIOAGRO in the Universidade Federal de Viçosa (Viçosa, Brazil). These strains were selected because *P. ostreatus* PLO06 has simple nutritional requirements and both strains show high rates of substrate decomposition. Stock cultures were kept stored in potato dextrose agar plates (PDA, Merck, Darmstadt, Germany) and subcultured every six months. Working cultures were also stored in PDA plates and maintained at 4°C until use.

Inoculum and substrate preparation

Inoculum of *P. ostreatus* PLO06 and *L. edodes* UFV73 was prepared by transferring a 8 mm disc from the working cultures to a new PDA plate followed by incubation for seven days at 25°C. The spawn was prepared in glass pots using pre-cooked and heat-sterilized rice grains (121°C). Spawn sterilization was performed three times for 2 h, during 48 h intervals. The spawn was inoculated

with approximately 10 agar disks (each with 1 cm in diameter) containing mycelia. The cultures were then incubated at 25°C for 20 days. After this incubation period, colonized rice grains were used (20 g samples) to aseptically inoculate polypropylene bags permeable to oxygen containing 1.5 kilograms of the heat-sterilized agro-industrial residues. Sterilization of the substrates used in this work was performed as described for the spawn.

P. ostreatus PLO06 was cultivated in eucalyptus bark, eucalyptus sawdust, corn cobs, sugarcane bagasse, coffee bark and coconut fiber while *L.edodes* UFV73 was grown in eucalyptus bark, eucalyptus sawdust, corn cobs and sugarcane bagasse supplemented with urea (0.5 %, w/w) to stimulate fungal growth. All the experiments were performed twice with at least two technical replicates.

Inoculated agro-industrial residues were kept in an incubation room maintained at room temperature (25°C). After the residues had been fully colonized (approximately 60 days), the polypropylene bags were transferred to a fructification room kept at 20°C and with relative humidity of 90% to allow the production of fructification bodies (mushrooms). Control treatments (substrates without fungal inoculation) were prepared and sterilized as described above for the inoculated agro-industrial residues. Control treatments and fructified substrates (obtained after harvesting the mushrooms) were transferred to paper bags (approximately 1.5 Kg) and dried at 60 $\pm$ 5 °C in an oven with forced ventilation. The residues were ground in a Willey mill, sieved through a mesh of 1 mm and 100 g of milled residue was packed individually in glass bottles of 200 g and stored at room temperature until being used for chemical and digestibility analysis.

Chemical analysis

After the samples were dried and milled, chemical analyses were performed to determine dry matter (DM), organic matter (OM), crude protein (CP), ether extracts (EE) and ash following the protocols described by AOAC (2000). The contents of neutral detergent fiber (NDF) were determined according to Mertens (2002), using thermostable amylase (Termamyl 120 L, Novozymes). The acid detergent fiber (ADF) and acid detergent lignin (sulfuric acid 72 %) were determined using sequential analyses proposed by Robertson and Van Soest (1981). The concentration of hemicelluloses was calculated as the difference between NDF and ADF performed sequentially on the same sample. Cellulose concentration was determined as the difference between ADF and ADL.

In vitro digestibility of neutral detergent fiber (IVDNDF)

The IVDNDF was determined by the method proposed by Tilley and Terry (1963), with some modifications, as follows. The ruminal fluid was collected (samples of 1000 mL) 2 h after feeding from cattle fistulated in the rumen and kept in the Dairy Cattle Facility of the Animal Science Department at the Federal University of Viçosa. Sampled animals graze on pasture and had *ad libitum* access to corn silage during the morning period. Ruminal digesta was filtered through four layers of gauze, stored in Thermo bottles and transported immediately to the laboratory, where the ruminal fluid was incubated at 39°C for about 30 min. Ruminal bacteria were collected anaerobically from the center of the flask.

The IVDNDF analysis was carried out in two stages. In the first stage, 350 mg samples of residues were weighed and mixed with 4 mL of rumen fluid in serum bottles followed by the addition of 32 mL of anaerobic McDougall buffer solution kept under CO_2 flux. The bottles were sealed with rubber stoppers and aluminum seals and were incubated at 39°C/120 rpm for 48 h. In the second step, following incubation for 48 h, the material was filtered in crucible filters that had been previously dried, weighed and washed three times with hot distilled water. After filtration, the crucibles were transferred to universal bottles and filled with 70 mL of neutral detergent solution. The bottles containing the crucibles were sterilized at 121°C for 15 min. After autoclaving, the crucibles were washed several times with hot water until the neutral detergent solution had been removed, followed by a final wash with 15 mL of pure acetone. The crucibles were transferred to the oven at 105°C until constant weight. All the analyses were performed twice in triplicate samples. The *in vitro* digestibility of neutral detergent fiber (%) was calculated based on the equations proposed by Tilley and Terry (1963).

Statistical analysis

All the experiments were performed with two biological replicates and triplicate samples. All the data regarding IVDNDF and sample chemical composition were subjected to analysis of variance (ANOVA) and significant differences were analyzed with the Tukey's test using the Statistical Analysis System and Genetics software (Ferreira, 2011). Differences among means with $P<0.05$ were considered statistically significant.

RESULTS

When lignocellulosic agro-industrial residues were inoculated with the white-rot fungus *P. ostreatus* PLO06, a decrease ($P<0.05$) in organic matter was often observed (Table 1). This effect was related with a greater mineralization of the organic matter, indicated by the increase ($P<0.05$) in ash concentration and greater *in vitro* digestibility of the fructified substrates (Tables 1 and 3). The only exception was the coconut fiber, where the fructified residue showed decreased mineralization (decrease in ash content). However, digestibility was still significant, which is coherent with the lower content of neutral detergent fiber (NDF) and acid detergent fiber (ADF) determined for this residue (Table 1).

Most inoculated residues also showed an increase ($P<0.05$) in the concentration of crude protein and ether extract, except for corn cobs in which differences in protein concentration and ether extract were not significant (Table 1). In the eucalyptus and coffee bark, non-inoculated and fructified residues had the same ether extract and crude protein content ($P>0.05$), respectively.

Except for the sugarcane bagasse, a greater degradation of NDF ($P<0.05$) was observed in the fructified residues, with an average decrease in fiber content of 20.57 % (Table 1). In sugarcane bagasse and corn cobs, hemicellulose was the main fraction hydrolyzed by *P. ostreatus* PLO06 ($P<0.05$), with lignin being little affected by the activity of the fungi (Table 1).

When eucalyptus bark and sugarcane bagasse were supplemented with 0.5 % urea, a decrease ($P<0.05$) in organic matter was observed in the fructified residues inoculated with *L. edodes* UFV73. In the corn cobs

Table 1. Chemical composition of lignocellulosic agro-industrial residues inoculated (1.33 % w/w) with the white-rot fungi *P. ostreatus* PLO06. Fructified residues (F) were inoculated with either *P. ostreatus* PLO06 and incubated until the production of fructification bodies (60 days).

Parameter	Eucalyptus sawdust		Eucalyptus bark		Coffee bark		Sugarcane bagasse		Corn cobs		Coconut fiber	
	NI	F	NI	F	NI	F	NI	F	NI	F	NI	F
	Composition (% in DM)											
DM	95.87^{a}	91.39^{b}	91.56	90.64	89.33	89.20	93.73	91.98	95.70^{a}	92.66^{b}	85.35^{b}	91.18^{a}
OM	98.04^{a}	93.80^{b}	93.38^{a}	89.41^{b}	94.55^{a}	91.57^{b}	97.54^{a}	92.95^{b}	97.58^{a}	94.45^{b}	95.17^{a}	98.37^{b}
CP	0.91^{b}	2.49^{a}	1.70^{b}	3.99^{a}	4.10	4.41	0.39^{b}	2.99^{a}	1.86	2.11	2.31^{b}	2.68^{a}
EE	0.23^{b}	1.77^{a}	0.65	1.43	0.08^{a}	2.41^{b}	0.24^{b}	4.22^{a}	1.84	1.27	1.34^{b}	9.54^{a}
NDF	91.45^{a}	76.47^{b}	83.65^{a}	65.71^{b}	75.56^{a}	57.96^{b}	57.67^{a}	64.6^{b}	83.54^{a}	65.81^{b}	94.13^{a}	74.79^{b}
ADF	85.37^{a}	66.80^{b}	76.61^{a}	56.25^{b}	67.97^{a}	47.81^{b}	41.79^{b}	50.66^{a}	46.53	46.81	87.66^{a}	69.99^{b}
CEL	50.80	48.25	50.70^{a}	41.55^{b}	40.94^{a}	36.48^{b}	32.31^{b}	45.75^{a}	38.69	40.32	34.52^{a}	28.99^{b}
HEM	6.75	9.66	6.74	8.65	7.58^{b}	10.15^{a}	17.56^{a}	13.95^{b}	36.93^{a}	19.00^{b}	6.38^{a}	4.08^{b}
ADL	25.53^{a}	19.00^{b}	17.17^{a}	7.22^{b}	24.09^{a}	15.35^{b}	6.27	7.03	8.48	7.43	46.38^{a}	36.30^{b}
Ash	1.95^{b}	6.19^{a}	6.62^{b}	10.58^{a}	5.45^{a}	8.43^{b}	2.46^{b}	7.04^{a}	2.4^{b}	5.55^{a}	4.82^{a}	1.62^{b}

Average followed by different letters in the same line for each residue differ at 5 % probability by Tukey's test. DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber; CEL = cellulose; HEM = hemicellulose; ADL = acid detergent lignin; NI = non-inoculated substrate; F = Fructified substrate.

Table 2. Chemical composition of lignocellulosic agro-industrial residues supplemented with urea (0.5 %, w/w) inoculated (1.33 % w/w) with the white-rot fungi *L. edodes* UFV73. Fructified residues (F) were inoculated with *L. edodes* UFV73 and incubated until the production of fructification bodies (60 days).

Parameter	Eucalyptus sawdust		Eucalyptus bark		Sugarcane bagasse		Corn cobs	
	NI	F	NI	F	NI	F	NI	F
	Composition (% DM)							
DM	85.94	85.34	90.82	89.06	91.95^{b}	93.29^{a}	93.84	89.18
OM	95.17^{b}	98.13^{a}	94.56^{a}	91.19^{b}	98.82^{a}	97.28^{b}	97.16	96.57
CP	3.74^{b}	4.09^{a}	4.00^{b}	7.15^{a}	2.58	3.29	3.04^{a}	5.21^{b}
EE	0.68	1.51	1.16	1.09	1.90	1.63	1.23	1.37
NDF	87.60	88.14	84.16^{a}	68.58^{b}	77.13^{a}	63.61^{b}	81.53^{a}	66.87^{b}
ADF	81.06	80.79	76.51^{a}	57.94^{b}	55.81	54.56	43.61^{a}	39.62^{b}
CEL	58.62	53.69	51.59^{a}	43.84^{b}	37.03	45.01	35.31^{a}	26.55^{b}
HEM	6.54	7.34	7.65	10.64	21.31^{b}	9.04^{a}	37.92^{a}	27.24^{b}
ADL	27.20	27.31	17.90^{a}	3.69^{b}	7.67	5.84	6.82^{b}	13.10^{a}
Ash	4.83^{a}	1.86^{b}	5.44^{b}	8.80^{a}	1.17^{b}	2.72^{a}	2.83	3.42

Average followed by different letters in the same line for each residue differ at 5 % probability by Tukey's test. DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber; CEL = cellulose; HEM = hemicellulose; ADL = acid detergent lignin; NI = non-inoculated substrate; F = Fructified substrate.

residue, differences ($P>0.05$) in chemical composition were not observed between the non-inoculated and fructified residues. The decrease in organic matter was directly related with the increase in ash content and this result was also observed for eucalyptus bark and sugarcane bagasse inoculated with *L. edodes* UFV73 (Table 2).

It was hypothesized that an increase in fungal biomass in the fructified residue could increase the concentrations of EE and CP in the residues. Except for the sugarcane bagasse, an increase in the concentration of CP ($P<0.05$) was observed in the fructified treatment of all residues. However, even in sugarcane bagasse a 27.5 % ($P> 0.06$) increase in CP was observed and the concentration of

Table 3. *In vitro* digestibility of neutral detergent fiber of non-inoculated (NI) or fructified (F) lignocellulosic agro-industrial residues. Fructified residues were inoculated with either *P. ostreatus* PLO06 or *L. edodes* UFV73 and incubated until the production of fructification bodies (60 days). Residues inoculated with *L. edodes* UFV73 were supplemented with urea (0.5 %, w/w).

Residue	NI	*F*	F/NI ratio
	***P. ostreatus* PLO06**		
Eucalyptus sawdust	15.36^{bD}	33.53^{aC}	2.2
Eucalyptus bark	17.05^{bD}	51.63^{aA}	3.0
Coffee bark	35.51^{bBC}	43.46^{aB}	1.2
Sugarcane bagasse	49.30^{bA}	56.03^{aA}	1.1
Corn cob	30.98^{bBC}	51.99^{aA}	1.7
Coconut fiber	13.99^{bD}	26.11^{aD}	1.9
	***L. edodes* UFV73**		
Eucalyptus sawdust	10.38^{C}	6.60^{C}	0.6
Eucalyptus bark	6.16^{bC}	53.91^{aB}	8.7
Sugarcane bagasse	44.14^{bA}	61.96^{aA}	1.4
Corn cob	29.31^{bB}	56.40^{aB}	1.9

Average followed by different lower case letters in the same line and followed by different capital letter in the columns differs at 5 % probability by Tukey's test. The statistical analysis was performed separately for each fungus. NI = non-inoculated substrate; F = Fructified substrate; F/NI ratio = Digestibility ratio between fructified and non-inoculated residues.

EE remained the same (P>0.05) for all the residues inoculated with *L. edodes* UFV73 (Table 2).

L. edodes UFV73 did not alter the chemical composition and digestibility of eucalyptus sawdust supplemented with 0.5% urea (Table 2). However, *L. edodes* UFV73 could alter the fiber composition of the agro-industrial residues eucalyptus bark and corn cobs, decreasing (P<0.05) major cell wall constituents (Table 2). In general, fructified residues had approximately 17.9% less NDF than non-inoculated residues and this effect also reflected in a lower ADF and cellulose content in most residues were the fungus was able to grow. Sugarcane bagasse was the only exception, since ADF and cellulose remained unchanged and hemicellulose contend reduced more than 57 % in the fructified residue (Table 2). Reduction in lignin content were only observed for eucalyptus bark, where the fructified residue showed a lignin content 79% lower than the non-inoculated substrate.

In vitro digestibility of neutral detergent fiber (IVDNDF) in the fructified residues was always greater than the non-inoculated substrate for *P. ostreatus* PLO06 (Table 3). Eucalyptus sawdust supplemented with 0.5% urea was the only residue treated with *L. edodes* UFV73 where digestibility was not affected (P>0.05). Digestibility of fructified eucalyptus bark inoculated with *P. ostreatus* PLO06 and *L. edodes* UFV73 increased approximately three-fold and eight-fold, respectively, compared to the non-inoculated substrate. The increase in digestibility varied from 13 to 200% for residues inoculated with *P. ostreatus* PLO06 and from 40 to 775% for residues inoculated with *L. edodes* UFV73.

Based on the fact that *P. ostreatus* PLO06 has simple growth requirements, substrate supplementation was not required for its growth in the lignocellulosic residues used in this work. However, *L. edodes* UFV73 could not develop well in residues with a high carbon/nitrogen ratio. Therefore, the supplementation of the residues with 0.5 urea or 20% rice bran (data not shown) was required for fungal growth. When the chemical composition and digestibility of agro-industrial residues that had been inoculated with *P. ostreatus* or *L. edodes* were compared, *P. ostreatus* showed a greater potential to improve the nutritional quality of the tested residues (Table 4). Increases in fiber degradation, protein content and digestibility were highly significant for both fungi, however, *P. ostreatus* was much better at degrading lignin and mineralizing the substrates (Table 4).

DISCUSSION

The white-rot fungi are among the most efficient microorganisms in depolymerization of complex substrates and lignin mineralization (Elisashvili et al., 2008; Sánchez, 2009). Edible mushroom are also highly appreciated for their nutritional, medicinal and gastronomic characteristics (Sánchez, 2010).

Table 4. Effect of white-rot fungi on chemical composition and digestibility of agro-industrial residues. Substrates inoculated with *P. ostreatus* or *L. edodes* were incubated for 60 days. Mushrooms were harvested (F) and the chemical composition and digestibility of the residues were compared with non-inoculated residues (NI).

Parameter	*P. ostreatus*		SEM	P	*L. edodes*		SEM	P
	NI	F			NI	F		
DM	91.92	91.17	0.71	0.471	90.64	89.22	0.56	0.115
OM	96.04[a]	93.42[b]	0.56	0.007	96.43	95.79	0.57	0.459
CP	1.88[b]	3.11[a]	0.21	0.001	3.34[b]	4.93[a]	0.28	0.003
EE	0.73[b]	3.44[a]	0.60	0.009	1.24	1.40	0.12	0.405
NDF	81.00[a]	67.56[b]	1.96	<0.001	82.61[a]	71.80[b]	1.65	0.017
ADF	67.66[a]	56.39[b]	2.45	0.007	64.25[a]	58.23[b]	1.84	0.048
CEL	41.33	40.22	1.55	0.626	45.64	42.27	1.71	0.203
HEM	13.66	10.91	1.55	0.239	18.36	13.57	1.69	0.081
ADL	21.32[a]	15.39[b]	0.92	<0.001	14.90	12.48	1.87	0.389
Ash	3.95[b]	6.57[a]	0.56	0.007	3.57	4.20	0.57	0.459
IVDNDF	27.03[b]	43.79[a]	3.61	0.003	22.49[b]	44.72[a]	7.21	0.046

Averages in the same line followed by different lower case letters for each fungus differs at 5 % probability by Tukey's test. DM = dry matter; OM = organic matter; CP = crude protein; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber; CEL = cellulose; HEM = hemicellulose; ADL = acid detergent lignin; IVDNDF = *in vitro* digestibility of neutral detergent fiber; NI = non-inoculated substrate; F = Fructified substrate.

Considering the growth of the mushroom market, there has been an increasing interest among farmers to produce mushrooms for commercial purposes using alternative lignocellulosic substrates (Sánchez, 2009). In this regard, the use of agro-industrial residues for mushroom production could be advantageous to reduce the environmental impact of these residues, to aggregate economical value into products of high nutritional value to humans (mushrooms) and to allow the use of such residues in animal rations.

In this work, we show that both *P. ostreatus* PLO06 and *L. edodes* UFV73 can alter the chemical composition and improve the digestibility of several agro-industrial residues of low nutritional value (Tables 1, 2, 3 and 4). We focused on the chemical composition and digestibility of the fructified residues (after the mushrooms being harvested) due to the fact that these substrates can be used for mushroom production directed to human consumption, which is a more profitable and noble destination for farmers.

Our results demonstrate that, on average, crude protein concentration was 56.5% greater after mushroom was harvested compared to non-inoculated agro-industrial residues (Table 4). In this regard, one could argue that if mushrooms are not destined to human consumption and the residues are used in animal rations with all the fungal biomass on it (including fruiting bodies), protein concentration could be much higher on these substrates. Additionally, other main components of the residues were also affected by fungal growth.

For *P. ostreatus* PLO06 and *L. edodes* UFV73, mineralization of most substrates was increased in the fructified residues, as indicated by greater ash content in the samples. The only exception was coconut fiber inoculated with *P. ostreatus* PLO06 and eucalyptus sawdust inoculated with *L. edodes* UFV73, which showed a reduction in ash content. *P. ostreatus* and *L. edodes* harbour many hydrolytic (CMCases, xylanases) and oxidative (laccases and maganese peroxidases) enzymes that decompose lignocellulosic biomass into low molecular weight molecules that can be further metabolized by the fungi. *P. ostreatus* usually grows well even in substrates with very low nutritional quality and high carbon/nitrogen ratios (Luz et al., 2012), whereas *L. edodes* has a high O_2 demand and requires more complex substrates for growth (Lee et al., 2012).

Losses of OM were also observed when the residues were inoculated with *P. ostreatus* PLO06 and *L. edodes* UFV73. The exception was coconut fiber inoculated with *P. ostreatus* PLO06 and eucalyptus sawdust inoculated with *L. edodes* UFV73. The result could be explained by a higher concentration of NDF, ADF and lignin in these residues, which was correlated with a decrease in fungal growth and reduced fruiting body formation (Gaitán-Hernández et al., 2011).

The white-rot fungi were able to degrade the structural and nonstructural components of different residues, reducing NDF, ADF and lignin. Analysis of the main fiber components cellulose, hemicelluloses and lignin indicated that cellulose and lignin were preferentially attacked by *P. ostreatus* PLO06 in eucalyptus sawdust, eucalyptus and coffee bark and coconut fiber. The relative increase in other plant cell components (cellulose) sometimes was observed (sugarcane bagasse) probably

due to the preferential utilization of soluble sugars and structural carbohydrates more accessible to enzymatic degradation, such as hemicellulose, in the residues colonized by the fungus. Elisashvili et al. (2008) evaluated the activity of lignocellulolytic enzymes produced by *L. edodes* and *Pleurotus* spp. strains in submerged and solid-state fermentation of various lignocellulosic wastes. The authors reported wide differences among the substrates tested and related enzyme activity to an increase in fungal biomass and the degradation of fibrous components of the substrate, including cellulose, hemicelluloses and lignin.

Lignin, a major component of plant cell wall of agro-industrial residues, reduced in residues treated with *P. ostreatus* PLO06 and even *L. edodes* UFV73 could reduce the lignin content of the residues in 16%. In contrast to *P. ostreatus*, the low nitrogen (N) content of lignocellulosic residues can be a limiting factor for *L. edodes* growth in agro-industrial residues. Therefore, supplemental nitrogen (urea or rice bran) is often added to lignocellulosic residues to stimulate carbon mineralization from accessible plant constituents (cellulose) as well as complex substrates, such as lignin.

Lignin is highly recalcitrant in the rumen, mainly due to the anaerobic nature of this ecosystem. Kilpatrick et al. (2000) showed that lignin is often associated with cellulose in a 3-D matrix that prevents the access of microbial extracellular enzymes to the cellulose in the plant cell wall. Different species of white-rot fungi produce hydrolytic enzymes with distinct kinetic properties and the efficiency of cellulose degradation can vary even among different fungal strains (Baldrian and Valásková, 2008). Although the white-rot fungi have the potential to decompose lignin and depolymerize lignocellulose, hemicellulose and cellulose, the chemical composition of the substrate affects the extension of substrate utilization and its applicability in ruminant feeds (Okano et al., 2006; Baldrian and Valásková, 2008; Rahman et al., 2011; Shrivastava et al., 2011; Tuyen et al., 2012; Mahesh and Mohini, 2013; Lynch et al., 2013; Tuyen et al., 2013).

The content of lignin decreased in all residues inoculated with *P. ostreatus*, except in the sugarcane bagasse and corn cobs. According to Allison et al. (2009), substrates with low nitrogen levels stimulate the degradation of lignin by white-rot fungi, while substrate with high concentrations of nitrogen promotes the degradation of structural polysaccharides (cellulose and hemicellulose).

Traditionally, supplementation of agro-industrial residues for the cultivation of *L. edodes* is performed to decrease the C/N ratio to approximately 20-25, since high concentrations of nitrogen can inhibit mycelia growth and prevent complete colonization of the substrate, as well as reduce the ligninolytic activity of the fungus. In this work, we observed that *L. edodes* UFV73 growth was limited to the surface of the substrate and a more thoroughly colonization was never observed on this substrate. Therefore, changes in other parameters could not be observed. Differences in degradation and digestibility of the main fiber components in the fructified residues inoculated with *L. edodes* UFV73 might be explained by the unique cell wall assembly and chemical structure of the lignin and lignin-carbohydrate complex in these residues.

Most residues inoculated with *P. ostreatus* PLO06 and *L. edodes* UFV3 increased the levels of CP and the content of EE. Gaitán-Hernández et al. (2006) showed that barley straw inoculated with *L. edodes* IE-105 had 35% more CP than untreated controls after 57 days of incubation, but a 44% decrease in CP was reported for wheat straw inoculated with a different strain of *L. edodes* and incubated for 61 days at 25°C. A similar pattern was reported for the levels of EE. The authors explained these contradictory results based on the differences in concentration of soluble carbohydrates between the substrates, suggesting that some variability in chemical composition should be expected among different lignocellulosic substrates treated with white-rot fungi.

Analysis of the i*n vitro* digestibility of neutral detergent fiber of fructified lignocellulosic residues (Table 3) revealed that the digestibility of lignocellulosic substrates inoculated with *P. ostreatus* PLO06 or *L. edodes* UFV3 was dramatically increased upon fungal fructification.

Differences for *in vitro* digestibility of neutral detergent fiber between residues inoculated with *P. ostreatus* PLO06 or *L. edodes* UFV73 (Table 3) can be explained considering two main factors: 1) differences in chemical composition of different substrates (Tables 1 and 2), in which the fibrous constituents (NDF, ADF and lignin) of plant cell walls are negatively correlated with digestibility (Velásquez et al., 2010); 2) differences in hydrolytic activity among the species of white-rot fungi used in the study. *P. ostreatus* and *L. edodes* are known to produce hydrolytic enzymes with distinct kinetic properties and the efficiency of cellulose degradation can vary even among different fungal strains (Baldrian and Valásková, 2008; Elisashvili et al., 2008).

Our data reveals that substrate digestibility is often correlated with the decrease in lignin content or an increase in crude protein concentration in the lignocellulosic residues, a characteristic that is highly desirable for the utilization of substrates in animal feeds (Okano et al., 2006; Rahman et al., 2011; Shrivastava et al., 2011; Tuyen et al., 2012; Mahesh and Mohini, 2013, Lynch et al., 2013; Tuyen et al., 2013).

Eucalyptus bark was always the residue with the highest increase in digestibility (Table 3), regardless of the species of white-rot fungi that was used to inoculate the substrate. Non-inoculated eucalyptus bark showed a high contend of minerals and substantial amounts of lignin and protein compared to other substrates, and these features of the chemical composition might have a significant effect on fungal growth and degradation of

insoluble substrates.

Based on these results, it appears that *P. ostreatus* PLO06 has advantages to improve the chemical composition and digestibility of low quality lignocellulosic agro-industrial residues, whereas *L. edodes* UFV73 would be advantageous for use in substrates with a more balanced carbon/nitrogen ratio. As such, *P. ostreatus* PLO06 has been recognized for its efficient biodegradation of different lignocellulosic substrates and more effective colonization of residues (Elisashvili et al., 2008; Luz et al., 2012), which could help us in preventing the growth of contaminants (saprophytic fungi) and improve its practical applications.

Conclusion

P. ostreatus PLO06 and *L. edodes* UFV73 were able to alter the physicochemical properties of major structural components contained in lignocellulosic residues so that they become more susceptible to degradation by rumen microorganisms. Overall, fructified substrates improved in protein concentration, showed lower fiber content and had greater *in vitro* digestibility of the neutral detergent fiber. The ash content revealed that lignin was being mineralized mainly by *P. ostreatus* PLO06, probably due to its pronounced oxidative activity. The use of lignocellulosic substrates for production of commercially valuable mushroom could be useful to aggregate biological value to residues that would be further incorporated into ruminant rations.

Conflict of Interest

The authors have not declared any conflict of interests.

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Arbuscular mycorrhizal fungi diversity in revegetated areas after bauxite mining

Wardsson Lustrino Borges[1]*, Cândido Barreto de Novais[2], Orivaldo José Saggin Júnior[3], Yves Prin[4], Christine Le Roux[4], Marc Ducousso[4] and Sergio Miana de Faria[3]

[1]Embrapa Amapá, Rodovia Juscelino Kubitschek, km 5, N°2600 CEP 68903-419 - Macapá, AP – Brasil.
[2]Universidade Federal de Lavras, DCS - Laboratório de Microbiologia do Solo, Caixa Postal 3037, CEP 37200-000, Lavras, MG, Brasil.
[3]Embrapa Agrobiologia, BR 465, km 07, CEP 23890-000, Seropédica, RJ – Brasil.
[4]CIRAD, UMR 82, Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM). Campus International de Baillarguet, Montpellier. France.

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Arbuscular mycorrhizal fungi (AMF) are obligatory biotrophs that have a symbiotic evolutionary relationship with about 80% of all terrestrial plant species. The fungus mainly supplies water and nutrients to the plant and receives photoassimilates. The AMF diversity affects both the competition among species and floristic composition of an area. Fluctuations in the population of this group of microorganisms can cause fluctuations in plant populations above ground. In this work, the AMF community profile in areas with different ages of revegetation was evaluated by morphological identification of spores and denaturing gradient gel electrophoresis (DGGE) techniques. We found 12 AMF species and dominance of the species *Glomus macrocarpum* determined by spore density. Since the richness level observed in each plot was low and there was predominance of one species of AMF, it can be concluded that these areas still present a high degree of disturbance. It was possible to detect complex band profiles by DGGE analyses for the two plant species studied, *Visnia latifolia* and *Cecropia hololeuca*. No relationship between AMF diversity and revegetation time was observed in these areas.

Key words: Denaturing gradient gel electrophoresis (DGGE), symbiosis, succession, survey.

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) belong to the Glomeromycota phylum. It is abundant and diverse in the soil of the majority of terrestrial ecosystems. These fungi are obligatorily biotrophic and establish mutualistic symbiotic relationships with the majority of land plant species, acting as an extension of the root systems

*Corresponding author. E-mail: wardsson.borges@embrapa.br. Tel: 55 (96) 40099512. Fax: 55 (96) 40099501.

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through the hyphae that they form. They influence the nutrition of plants through two basic mechanisms: by increasing the root absorption surface and increasing the soil volume exploited by the root system. These mechanisms increase the absorption of nutrients, especially those that have lower mobility in the soil, such as phosphorus, and facilitate the uptake of water under drought conditions.

The beneficial effects of AMF for many plant species have been reported in the literature, both for agricultural crops like *Manihot esculenta* and forest species such as *Trema micrantha*, *Schinus terebinthifolius*, *Senna macranthera* and *Caesalpinia ferrea* (de Souza et al., 1999; Siqueira and Saggin-Júnior, 2001). AMF have a synergetic effect on biological nitrogen fixation (BNF) in some triple interactions (fungus-plant-bacteria), increasing this fixation because leguminous species are better nourished in association with AMF or when the fungi influence the radicular infection process (Jesus et al., 2005).

Recently the role of AMF in complex ecosystems has been studied (Siqueira et al., 1998; Zangaro et al., 2007; 2008). Besides the benefits mentioned above, these fungi increase plants' tolerance for toxic elements, which is particularly important in contaminated environments. For this reason, they are fundamental to improve the survival of seedlings in mined areas in the process of revegetation (Chaer et al., 2011; Soares and Siqueira, 2008).

The diversity of AMF has been evaluated by identifying morphological characteristics of their spores or by using molecular biology tools. Some authors urge the use of these tools because the population of spores present in the soil may have little relationship with the AMF colonizing the roots (Husband et al., 2002a, b). In this case, improved molecular techniques can help directly identify the AMF population in root systems

The aim of this work was to assess the AMF diversity using morphological characterization of the spores obtained from soil samples and to evaluate the profile of the AMF community colonizing the roots of *Visnia latifolia* and *Cecropia hololeuca* by means of denaturing gradient gel electrophoresis (DGGE), in areas with different revegetation ages after bauxite mining.

MATERIALS AND METHODS

Study area

The company Mineração Rio do Norte (MRN) operates the Saracá, Almeidas and Avisos mines (all within the Saracá-Taqüera National Forest, located in the municipality of Oriximiná, Pará state, at 1º 21' S - 56º 22' W, 180 m elevation). In these mines, the ore is found at an average depth of 8 m, covered by dense vegetation and a layer called overburden, composed of organic soil, nodular bauxite and ferruginous laterite. To mine the reserves, it is necessary to remove the overburden to reveal the economically exploitable bauxite ore. This operation is done in sequence, where the overburden is deposited in the adjacent pit that was previously mined. In these areas, the replanting is done on the overburden. The replanting of the overburden areas investigated in this study was done by the company using available seeds of various species (*Parkia multijiga*, *Parkia pendula*, *Parkia oppositifolia*, *Ormosia holerytra*, *Ormosia excelsa*, *Sclerolobium paniculatum* and *Acosmium nitens*) in 1982, 1983, 1984, 1985, 1986, 1987, 1992, 1993, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005 and 2006.

Evaluation of the AMF community by morphological characteristics of spores

Twenty simple samples (0 – 0.20 m) were harvested to compose a compound sample in each plot measuring 250 m^2 in each area. Thirty-two compound soil samples were collected in 2007. For the years 1987, 2004, 2005 and 2006 soil samples were not collected because in these years only one plot was revegetated.

Spores were extracted from 50 mL soil samples by wet sieving and decanting (Gerdemann and Nicolson, 1963) then centrifuging in water and 45% sucrose solution (Jenkins, 1964). The spores recovered were counted and grouped according to their size and color. Representatives of each group were mounted on slides for microscopic examination using polyvinyl-lacto-glycerol (PVLG) and PVLG plus Melzer reagent (1:1) as mounting. The AMF species were identified by comparison with descriptions found in Schenck and Perez (1990) and at the site of the International Culture Collection of Arbuscular Mycorrhizal Fungi (http://invam.caf.wvu.edu).

The species richness, shannon-weiner diversity and evenness (J') values were determined using the Past program (PAlaeontological STatistics) (Hammer et al., 2001).

Evaluation of the AMF community by DGGE

Twenty-nine and 17 root samples of *V. latifolia* and *C. hololeuca* were collected, respectively. These species were chosen because of the native occurrence in the region and are often found in areas in the early stages of succession. Specimens of *V. latifolia* were found in areas revegetated in 1982, 1984, 1986, 1987, 1999, 2004 and 2006 (1 individual for each year); 1993, 1995, 1996, 1997, 1998, 2000, 2001, 2003 and 2005 (2 individuals each) and 2002 (4 individuals). In turn, *C. hololeuca* specimens were found in areas revegetated in 1986, 1998, 2005 and 2006 (1 individual each); 1999, 2002 and 2003 (3 individuals each); and 2001 and 2004 (2 individuals each). The roots were dehydrated in silica gel for the transport and then stored in 80% ethanol (v/v) at 4ºC in the laboratory. Before DNA extraction, some samples were randomly selected to confirm the root colonization and all samples showed fungal structures.

For DNA extraction, the root samples were taken from alcohol, dried quickly on absorbent paper at 65ºC to eliminate residual alcohol and macerated in liquid nitrogen. The DNA extraction was done with the DNeasy Mini Plant kit (Qiagen). The 18S rDNA gene was amplified, in two PCR reactions. In the first PCR, the primers used were AML1 (5'-ATCAACTTTCGATGGTAGGATAGA-3') and AML2 (5'-GAACCCAAACACTTTGGTTTCC-3') (Lee et al., 2008). The final volume of the reaction was 15 μL and the amplification conditions were: 1X buffer; 200 μM of each dNTP; 1.625 mM of $MgCl_2$; 0.2 μM of each primer; 0.14 μg μL^{-1} of BSA (bovine serum albumin); 0.78% v/v of formamide and 1 U of DNA polymerase (GoTAq® Flexi DNA Polymerase - Promega). Two μL of a 1:20 dilution of the extraction product was used as template DNA. The reaction was carried out in an Eppendorff thermocycler with the

following steps: 94ºC for 3 min for initial denaturing; 30 cycles at 94ºC for 45 s for denaturing; 58.5ºC for 1 min for annealing; 72ºC for 55 s for extension; and 72ºC for 5 min for final extension.

The PCR product was verified in 1% (w/v) agarose gel. For the samples presenting visible bands, a 1:1000 dilution was made in water, while the dilution for the other samples was 1:500.

The second reaction used the primers NS31 (5'-TTGGAGGGCAAGTCTGGTGCC-3', Simon et al., 1992), containing the GC clamp NS31GC (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGT TGGAGGGCAAGTCTGGTGCC-3', Kowalchuk et al., 2002), and AM1 (5'- GTTTCCCGTAAGGCGCCGAA-3', Helgason et al., 1998). In this reaction, the final volume was 35 µL and the amplification conditions were: 1X buffer; 200 µM of each dNTP; 2.5 mM of $MgCl_2$; 0.2 µM of each primer; 0.135 µg μL^{-1} of BSA; and 1 U of DNA polymerase (GoTAq® Flexi DNA Polymerase - Promega). The template DNA consisted of 2 µL of the dilution of the first PCR product. The reaction was performed in the same thermocycler, with the following steps: 94ºC for 3 min for initial denaturing; 34 cycles at 94ºC for 1 min for denaturing; 60ºC for 1 min for annealing; 72ºC for 1 min for extension; and final extension at 72ºC for 5 min.

The products obtained with the primers NS31GC and AM1 were separated by polyacrylamide gel (6% w/v) electrophoresis (37.5:1 acrylamide:bisacrylamida) with a chemical denaturing gradient composed of formamide and urea, varying from 30 to 45% (100% denaturant corresponds to 7M of urea and 40% formamide), in 0.5X TAE at 60ºC under constant voltage of 75 V for 15 h. The gels were stained with ethidium bromide and photographed under UV light.

The band profiles were used to construct similarity dendrograms with the Jaccard index and the UPGMA clustering method, available in the GelCompar II program (Applied Maths).

RESULTS

Evaluation of the AMF community by spore occurrence

Twelve AMF species were identified, one of them belonging to the *Glomus* genus (*G. macrocarpum*), one to the *Claroideoglomus* (*C. etunicatum*), one to the *Scutellospora* (*S. scutata*), one to the *Gigaspora* (*G. margarita*), three to the *Rhizophagus* (*Rhizophagus* sp., *Rhizophagus clarus*, *Rhizophagus fasciculatus*) and five to the *Acaulospora* genus (*A. foveata*, *A. mellea*, *A. scrobiculata*, *A. tuberculata* and *Acaulospora* sp.).

G. macrocarpum spores were found in all the soil samples and these spores accounted for 97% of all the spores found. Besides this, *G. macrocarpum* was the only AMF species found in eight of the soil samples and the only one found in the samples from the plots revegetated in 1995 and 2000. Spores of this species represented at least 82% of the total for each year analyzed (Figure 1A). *A. mellea*, *G. margarita* and *S. scutata* were found in 11, 8 and 7 soil samples, respectively, while *A. scrobiculata* and *R. clarus* were only found in one sample each.

The mean species richness varied between 1 and 4 and was higher in the areas revegetated in 1982, 1983, 1993, 1997, 1998, 2001, 2002 and 2003 (Figure 1B). The Shannon index and evenness index values were higher in the plots revegetated in 1983, 1985, 1998, 2002 and 2003 (Figure 1B).

Evaluation of the AMF community by DGGE

The number of bands ranged from 2 to 5 for *C. hololeuca* and 0 to 7 for *V. latifolia* by the DGGE technique. For *V. latifólia* there were two principal clusters formed both by DGGE (Figure 2). The DGGE analysis revealed that the two samples from 1998-A1 (1 and 2); 1997-A1-1 and 1997-A2-1; 1995-A2-1 and 1987-A2-1; 2001-A1-1 and 2002-A1-1; 2000-A2 (1 and 2) and 1996-A13-1 presented 100% similarity (Figure 2). For *C. hololeuca,* two samples from 2003-02 (1 and 2); two 2004-FP samples (1 and 2); 1999-A2-2 and 2001-A2-2; 2002-A1-3 and 1986-A1-1; and 1999-A2-1 and 2001-A1-1 presented 100% similarity (Figure 3).

DISCUSSION

The predominance of spore and AMF species belonging to the *Acaulospora*, *Rhizophagus* and *Glomus* genera observed in this study is in accord with other studies in various ecosystems (Carrenho et al., 2001; Leal et al., 2009; Lovelock et al., 2003), including studies of reclaimed mining areas (Caproni et al., 2003, 2005; Mergulhão et al., 2010).

Caproni et al. (2003) identified 57 species, belonging to six genera, with the greatest number belonging to the *Glomus* genus, followed by *Acaulospora*, *Entrophospora, Scutellospora, Gigaspora* and *Archeospora*. Caproni et al. (2005) identified a total of 18 AMF species belonging to the genera *Glomus* (7 species), *Acaulospora* (6 species), *Scutellospora* (2 species), *Archeospora*, *Entrophospora* and *Gigaspora* (1 species each).

Carrenho et al. (2001) evaluating the effect of corn monoculture for three consecutive years on the composition of the AMF community, reported that *G. macrocarpum* was recovered from 100, 90 and 60% of the samples in the first, second and third years, respectively. Caproni et al. (2003) found *G. macrocarpum* in 93% of the samples analyzed. According to them, the high frequency and relative density observed for this species indicate a wide range of adaptation. They stressed that it is important to study more details of the ecology of this species because it has potential for use in programs to restore degraded areas.

The diversity of AMF species can be influenced by the composition of plant species and environmental and edaphic factors (Carrenho et al., 2001; Mergulhão et al., 2009). This fact can also be related to the characteristics inherent to the life strategy of these species. Species of these genera usually produce small spores, in great

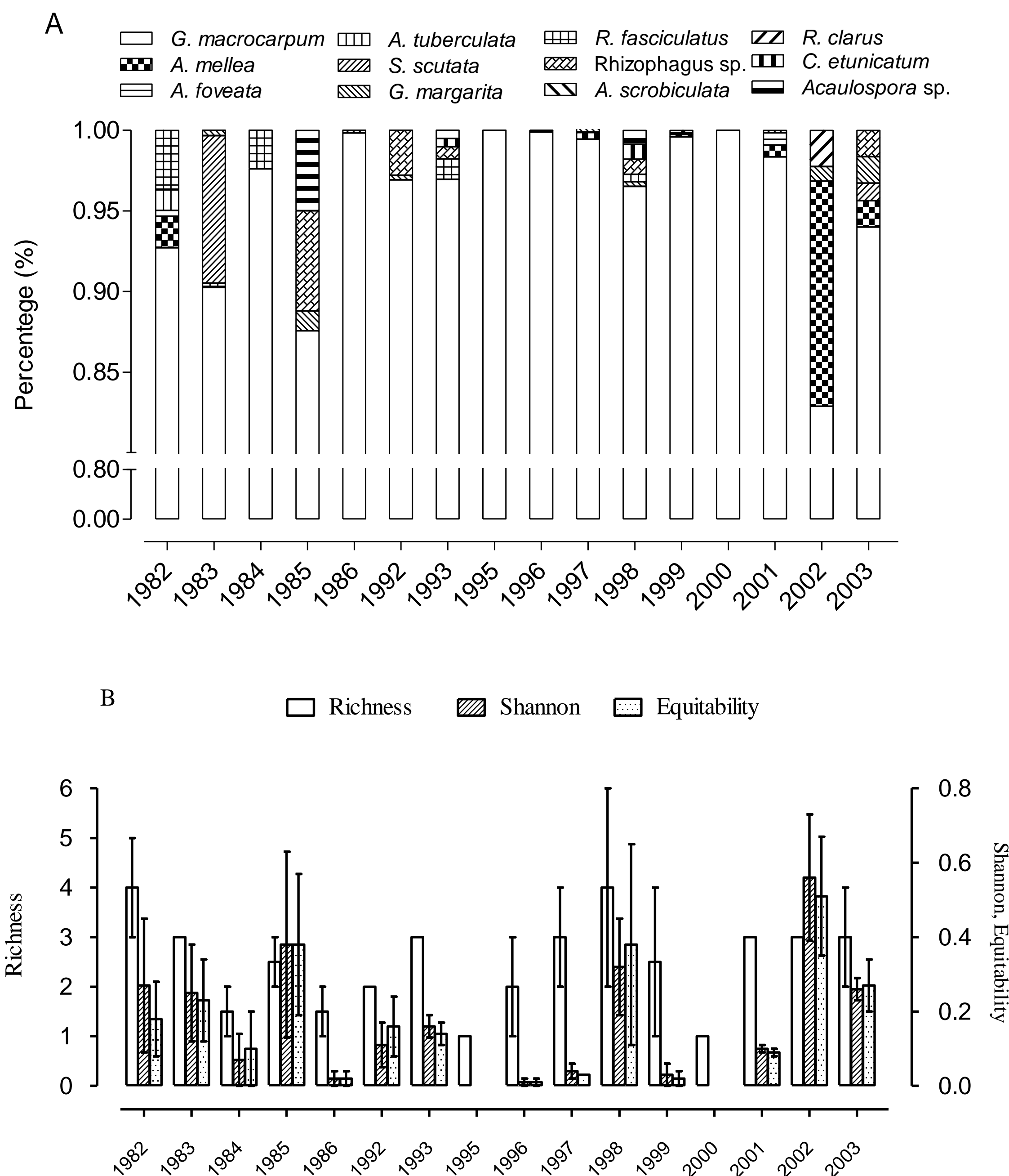

Figure 1. **A.** Percent of spores of each AMF species. **B.** mean and standard error for species richness, Shannon index and evenness index in function of year of revegetation.

abundance, and colonize the roots plentifully, while *Gigasporaceae* species generally produce larger spores, in smaller number, with less intense colonization.

Although there are limitations to the technique, some authors have used the number of bands in DGGE gel as an estimate of the species richness (Zhang et al., 2009). Considering that each band in the gel represents an AMF taxon, there was greater richness of the samples from the areas planted with *V. latifolia* than in the samples from the areas planted with *C. hololeuca*. This situation can indicate differences in the degree of specificity of these species in relation to mycorrhizal colonization.

The DGGE analysis indicated 100% similarity between some root samples (Figures 2 and 3). Interestingly, in some cases, the samples were from the same revegetated plot and other cases where they were collected

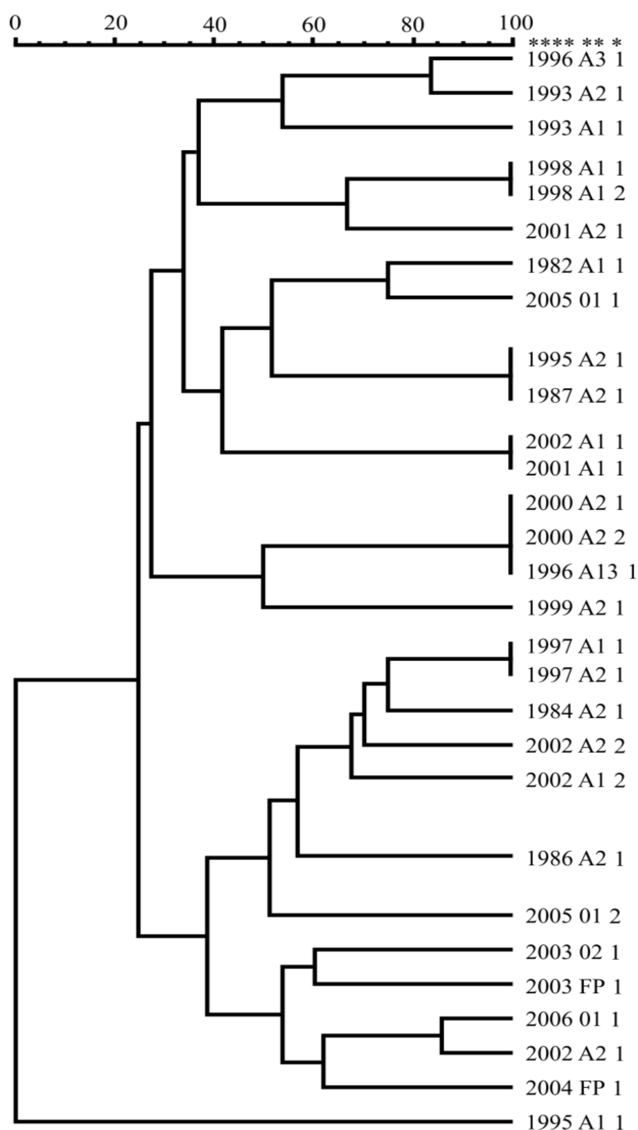

Figure 2. Genetic similarity dendrograms of the AMF community for samples from roots of *Visnia latifolia* evaluated by denaturing gradient gel electrophoresis (DGGE) of the 18S rDNA gene. **** Year of revegetation; ** Plot sampled; * Plant specimen.

from different plots.

Melloni et al. (2003) concluded that in areas being restored after mining, the diversity of AMF species is more closely related to the type of vegetation introduced than to the time since revegetation. In the present study we did not observe any relationship between the profile of the AMF community, analyzed both by spore presence and by molecular tools, and time since the areas were revegetated. We also did not observe any progressive alteration of the relative density of the different species in function of time revegetation, which if present would have characterized succession within the AMF community.

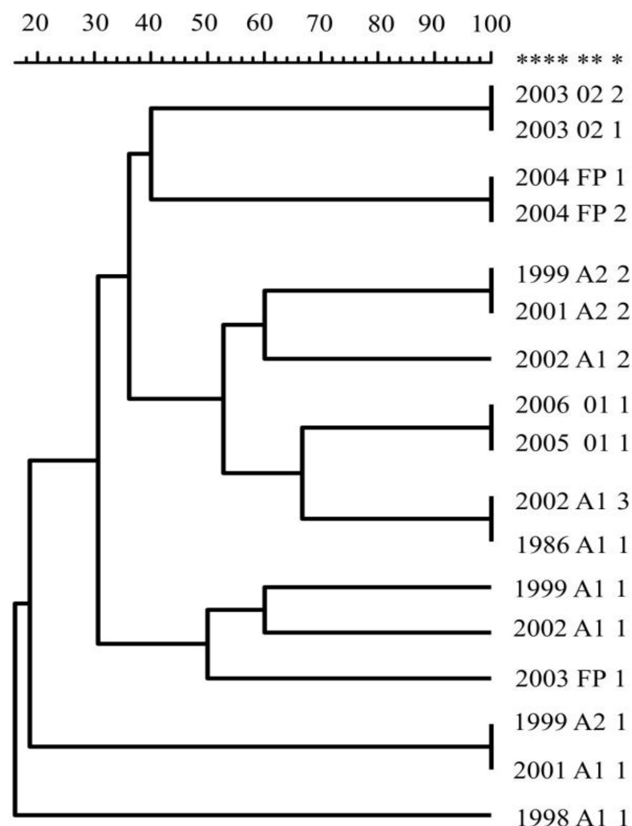

Figure 3. Genetic similarity dendrograms of the AMF community for samples from roots of *Cecropia hololeuca* evaluated by denaturing gradient gel electrophoresis (DGGE) of the 18S rDNA gene. **** Year of revegetation; ** plot sampled; * plant specimen.

The results obtained show that the AMF community in the areas analyzed might be determined by varied combinations of environmental and edaphic factors; composition of plant species; spatial variability of the AMF community; and specificity of the host species in each area analyzed.

In general, the results show that despite the degree of degradation caused by mining activity, the revegetation practices followed are enabling the reestablishment of the AMF community in the soil. However, the areas studied still present a high level of disturbance, because the AMF richness and number of spores found were both low and there was overwhelming predominance of a single species, *G. macrocarpum*.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Isolation and characterization of *Escherichia coli* O91:H21 in a sample obtained from cattle

Marcela A. Pizarro[1]*, Juan H. Orozco[1], S. M. Degarbo[1], R. Silva[1], A. Laciar[2] and M. E. Rüttler[1]

[1]Department of Morphophysiology, Laboratory of Molecular Biology, Faculty of Medical Sciences, National University of Cuyo, Av Libertador 80 (5500) Mendoza, Argentina.
[2]Cátedra of Microbiology, Faculty of Biochemistry, Chemistry and Pharmacy. National University of San Luis, Argentina.

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The *Escherichia coli* Shiga toxin (Stx) producing strains may cause medical conditions that range from diarrhea to hemolytic uremic syndrome; life-threatening conditions worldwide. Shiga toxin-producing *E. coli* (STEC) strains isolated from patients who usually possess, in addition to one or more *stx* genes, the *eae* gene, encoding adhesin intimin. However, a subset of STEC strains associated with human disease lack *eae*. One of the most common among these is the serogroup O91. This paper reports on the isolation and description of the genophenotypic characteristics from a bovine-derived *E. coli* O91:H21 strain in a slaughterhouse dependent from Cattle Direction of Mendoza Province, during 2007.

Key words: *E. coli* O91:H21, hemolitic uremic syndrome (HUS), diarrhea, cattle.

INTRODUCTION

Shiga toxin-producing *E. coli* (STEC) has been identified as the causative agent of foodborne diarrhea since 1982. STEC strains are characterized by the production of one or more types of cytotoxins which cause tissue damage in humans and animals. On the basis of their pathogenicity in humans, a subset of STEC strains are classified in the category of Enterohemorrhagic *E. coli* (EHEC). Symptoms of EHEC infection in humans range from abdominal pain and watery diarrhea and hemorrhagic colitis to hemolytic uremic syndrome (Paton and Paton, 1998; Riley et al., 1983; Verweyen et al., 2000).

The natural reservoirs of STEC are domestic animals and wild ruminants, which eliminate the bacteria in their stool, disseminating them in the environment (Caprioli et al., 2005). STEC-infected animals usually do not show signs of infection and can be included in the food production chain. As a result, animal products such as meat or milk, pose a risk of contamination with STEC from such animals (Mellmann et al., 2008). Consumption of STEC contaminated food has been identified as the most important route for human infection with these pathogens (Caprioli et al., 2005; Mellmann et al., 2008).

Typical EHEC strains isolated from patients have, besides the encoding cytotoxins genes mentioned above, the eae gene that encodes the adhesin intimin (Bettelheim, 2007; Bielaszewska et al., 2006). However, a subgroup of EHEC strains associated with disease in humans lacks the *eae* gene, thus denominated atypical EHEC (Bettelheim, 2007; Karmali et al., 2003; Pradel et al., 2008; Pulz et al., 2003).

*Corresponding author. E-mail: mpizarro@fcm.uncu.edu.ar.

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Table 1. Oligonucleotides used in the PCR reactions.

Primer	Primer sequence (5'-3')	Size bp	Reference source
Stx1a	GAAGAGTCCGTGGGATTACG	130	Pollard et al., 1990
Stx1b	AGCGATGCAGCTATTAATAA		
Stx2a	TTAACCACACCCCACCGGGCAGT	346	Pollard et al., 1990
Stx2b	GCTCTGGATGCATCTCTGGT		
eae a	TGAGCGGCTGGCATGAGTCATAC	240	Pass et al., 2000
eae b	TCGATCCCCATCGTCACCAGAGG		
astA a	GCCATCACA GTA TAT CCG	108	Ruttler et al., 2006; Schmidt et al., 1995
astA b	GCG AGT GAC GGC TTT GTA GT		
*Hly*A1	GGTGCAGCAGAAAAAGTTGTAG	1551	Schmidt et al., 1995
*Hly*A4	TCTCGCCTGATAGTGTTTGGTA		
SAAD F	CGTGATGAACAGGCTATTGC	119	Paton and Paton, 2002
SAAD R	ATGGACATGCCTGTGGCAAC		
AggR F	AGACGCCTAAAGGATGCCC	430	Ruttler et al., 2006
AggR R	GAGTTATCAAGCAACAGCAATGC		

Whereas EHEC O157:H7 was known to be the predominant serotype associated with most of the documented EHEC-related outbreaks worldwide, previous epidemiological studies imply that non-O157 EHEC infection can become problematic for public health (Caprioli et al., 2005). The non-O157 EHEC strains associated with human disease include the serotypes O8:H-, O26:H11, O91:H21, O103:H2, O111:H-, O113:H21 and O128:H2 (Padola et al., 2002).

E. coli O91: H21 is a common serotype among EHEC eae negative subset. The strains belonging to this serotype have been classified within the seropathotype "C" to cause disease in humans, although with low incidence and low association with outbreaks, are known to be highly virulent (Bielaszewska et al., 2006; Bugarel et al., 2010; Kim et al., 2013; Kruger et al., 2006; Mellmann et al., 2008; Mellmann et al., 2009). The strains within this serogroup appear to be transmitted predominantly by food, because food vehicles have been identified as the only risk factors for adults with sporadic STEC O91 infection in Germany; O91 is the second most frequently isolated STEC serogroup in routine food samples in that country, and O91 is the only major STEC serogroup with no association between incidence of human infection and cattle density. Despite frequent isolation of STEC O91 from humans, the clonal relatedness of the serotypes of this serogoup is poorly understood (Mellmann et al., 2009).

Although this serotype has been isolated previously in Argentina in cattle, and its strains have been phenotypically and genotypically described (Kruger et al., 2006), there are no reports of strains belonging to this serotype until now, from either animal or human samples in Mendoza. This work reports the finding of a bovine-derived *E. coli* O91:H21 strain in a slaughterhouse in the Directorate of Livestock in the province of Mendoza in 2007.

MATERIALS AND METHODS

This isolation carries identified pathogenicity factors that enhance its virulence. So far, there has not been reported illness in patients associated with this serotype in our city.

This strain was found in the gut content of a freshly slaughtered animal intended for consumption. Detection was performed by PCR, looking for strains carrying Stx2 genes, and then the isolation of the strain was carried out following the methodology described: 91 rectal swabs of 91 animals intended for slaughter and 108 plating samples from the carcasses of 50 of them were analyzed during a period of nine months in 2007.

The samples were taken according to the rules and regulations set forth by SENASA, the organization that controls agriculture and livestock farming in Argentina. This procedure was carried out during the anal enucleating of animals.

PCR was carried out as a screening technique, looking for strains carrying encoding Shiga toxin1 (Stx1), and Shiga toxin 2 (Stx2) genes (Paton and Paton,1998). A total of 199 (91 rectal swabs and 108 carcass) samples were studied. The reactions were carried out in 50-µL reaction volumes containing 2 µL of bacterial lysate, 200 mmol of deoxynucleotide mix (dATP, dTTP, dCTP, and dGTP), 100 pmol of each primer, 5 µL of 10XPCR buffer with 1.5 mM $MgCl_2$, and 2.5 U of *Taq* DNA polymerase (InbioHighway).

After the screening by PCR, the positive broths were reseeded on plates, and then pools of 10 colonies were tested again until the colony carrying the gene was found. Then, the E.*coli* strain was identified by standard biochemical tests as oxidase negative, indole positive, Simon's citrate negative, urease negative, and hydrogen sulfide negative (MacFaddin, 2003). Once the strains were isolated and reconfirmed by PCR, their genotyping was conducted by identification of coding genes of these proteins: intimin (eae) (Pass et al., 2000), enterohemolysin (HlyA) (Schmidt et al., 1995), enteroaggregative thermoestable toxin EAST1 (*astA*) (Ruttler et al., 2006; Schmidt et al., 1995) STEC binding adhesin (*Saa*) (Paton and Paton, 2002) (Table 1). the detection of Stx1 and Stx2 genes was carried out in a mutiplex reaction, while the remaining reactions

were performed in a single reaction. The following strains were used as positive controls: *E. coli* EDL 933 (O157: H7, Stx1 +, Stx2 +, *eae* +, *astA* +), *E. coli.* O157: H7 *Hly* +, (Strains provided by National Institute of Infectious Diseases "Dr. Carlos G. Malbrán); *E. coli saa* + (strain provided by Dr. Paula Lucchesi, Faculty of Veterinary Medicine, National University of Centro, Tandil, Buenos Aires, Argentina).

The reactions were performed in a thermocycler Eppendorf Mastercycler Personal, as previously described protocols (Pollard et al., 1990; Schmidt et al., 1995; Pass et al., 2000; Rüttler et al., 2006; Paton and Paton, 2002).

For detection of sero - group, agglutination was conducted with monoclonal antisera anti-O and anti-H at the Veterinary Medicine School, Buenos Aires Central National University, at Tandil City. The strain being studied agglutinated with antisera anti O91:H21.

Vero cells have a high sensitivity to Shiga toxins and trial on cytotoxicity using this cell line is the "gold standard" technique as control strains were used; *E. coli* O157: H7 C984 (Stx1 +) and *E. coli* O157: H7 from 1271-84 (Stx2+) (Strains provided by National Institute of Infectious Diseases "Dr. Carlos G. Malbrán). The cytotoxity trial was performed as previously described (Karmali et al., 1985) on Vero cells (passage 8) cultured with Mi nimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and gentamicin (50 µg/mL). In order to achieve convergence, the required cell concentration was used.

RESULTS AND DISCUSSION

Twelve strains of *E. coli* were identified as having at least one virulence factor present in *E. coli* pathogenic. It was possible to recover only one strain O91: H21 from a sample of intestinal contents.

The strain confirmed as *E. coli* O91: H21 carried the genes encoding Stx2 toxin, the enterohemolysin (*HlyA*), and STEC binding adhesin *(Saa).*

Cytotoxic activity was estimated in *E. coli* O91:H21 Stx2+ strain, considering the reverse title of highest dilution provided a 50% destruction of the monolayer as a cytotoxic unit (DC50) (Basta et al., 1989; Karmali, 1989) Strain *E. coli* O91: H21 showed marked cytotoxic effect up to 1:64 dilutions.

It also turned out to be negative for the gene encoding the synthesis thermostable toxin enteroaggregative *E. coli* (*ast*A gene) that is present in some EHEC strains as well (Savarino et al., 1996).

It is known that most of the strains isolated from human origin, have common properties directly related to virulence, including hemolysin production, induction of injury "attaching and effacing" in intestinal epithelial cells and production of toxins Stx1 and Stx2. Therefore, to predict whether the isolates from animal sources are potentially pathogenic to humans, it is necessary to determine the presence and the virulence factor profile (Paton and Paton, 1998; Bielaszewska et al., 2006).

Moreover, recent "Enter-Net" data, a global consortium of enteric disease surveillance of 35 countries, showed that the number of cases of disease caused by non-O157 EHEC increased by 60.5% compared to 13% increase in cases involving EHEC O157 (Pihkala et al., 2012).

In other works consulted, similar results were obtained with respect to the phenotypic characteristics of strain *E. coli* O91: H21 in this study (Mellmann et al., 2008; Mellmann et al., 2009). This strain could be considered as atypical EHEC that encode the synthesis of Shiga Toxin 2, enterohemolysin STEC Binding adhesin but not for intimin.

Based on the fact that Shiga toxin type 2 (Stx2) is primarily responsible for renal failure in the HUS, extremely hygienic-sanitary controls are necessary for cattle products, especially beef, so they do not pose a risk to the population.

These data highlight the importance of studying these potentially pathogenic strains and their comprehensive characterization to be alert to the existence of virulent strains circulating in the population's food chain. The information will also facilitate the comparison of the characteristics of the animal strains with those isolates obtained from patients with clinical symptoms, for the purpose of defining the potential pathogenic strains of this serotype isolated in the region.

The implementation of prevention and control strategies in public health impact are essential to reduce morbidity and mortality associated with HUS, as emphasizes the World Health Organization. Therefore, it is necessary to improve pathogen control measures through the agro-food chain to ensure food quality. Education programmes should be aimed to the community in general, warning about the risks of this agent, its ways of transmission as well as to implement prevention strategies.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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