African Journal of Microbiology Research

Volume 9 Number 15, 15 April, 2015
ISSN 1996-0808

Academic Journals

ABOUT AJMR

The **African Journal of Microbiology Research (AJMR)** (ISSN 1996-0808) is published Weekly (one volume per year) by Academic Journals.

African Journal of Microbiology Research (AJMR) provides rapid publication (weekly) of articles in all areas of Microbiology such as: Environmental Microbiology, Clinical Microbiology, Immunology, Virology, Bacteriology, Phycology, Mycology and Parasitology, Protozoology, Microbial Ecology, Probiotics and Prebiotics, Molecular Microbiology, Biotechnology, Food Microbiology, Industrial Microbiology, Cell Physiology, Environmental Biotechnology, Genetics, Enzymology, Molecular and Cellular Biology, Plant Pathology, Entomology, Biomedical Sciences, Botany and Plant Sciences, Soil and Environmental Sciences, Zoology, Endocrinology, Toxicology. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles are peer-reviewed.

Submission of Manuscript

Please read the **Instructions for Authors** before submitting your manuscript. The manuscript files should be given the last name of the first author

Click here to Submit manuscripts online

If you have any difficulty using the online submission system, kindly submit via this email ajmr@academicjournals.org.

With questions or concerns, please contact the Editorial Office at ajmr@academicjournals.org.

Editorial Board

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

Instructions for Author

Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

The **cover letter** should include the corresponding author's full address and telephone/fax numbers and should be in an e-mail message sent to the Editor, with the file, whose name should begin with the first author's surname, as an attachment.

Article Types

Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.

Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

Reviews: Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Reviews should be concise and no longer than 4-6 printed pages (about 12 to 18 manuscript pages). Reviews are also peer-reviewed.

Review Process

All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review.

Decisions will be made as rapidly as possible, and the Journal strives to return reviewers' comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the AJMR to publish manuscripts within weeks after submission.

Regular articles

All portions of the manuscript must be typed double-spaced and all pages numbered starting from the title page.

The Title should be a brief phrase describing the contents of the paper. The Title Page should include the authors' full names and affiliations, the name of the corresponding author along with phone, fax and E-mail information. Present addresses of authors should appear as a footnote.

The Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length.. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.

Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

A list of non-standard **Abbreviations** should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

References: In the text, a reference identified by means of an author‘s name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author‘s name should be mentioned, followed by ’et al‘. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ’a‘ and ’b‘ after the date to distinguish the works.

Examples:

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)
References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. Afr. J. Biotechnol. 7: 3535-3539.

Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

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

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.
Proofs and Reprints: Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.

Fees and Charges: Authors are required to pay a $550 handling fee. Publication of an article in the African Journal of Microbiology Research is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances

African Journal of Microbiology Research

Table of Content: Volume 9 Number 15, 15 April, 2015

ARTICLES

Arbuscular mycorrhizal fungi in the *Jatropha curcas* rhizosphere
Bruno Coutinho Moreira, Ana Lúcia Rodrigues, Sabrina Feliciano Oliveira, Paulo Sérgio Balbino Miguel, Denise Mara Soares Bazzolli, Sidney Luiz Stürmer and Maria Catarina Megumi Kasuya

The role of phenolic compounds in the defense of sooty mold of olive leaves (*Olea europea* L.)
Faiza Ilias, Sarra Bensehaila, Kenza Medjdoub, Imad El Haci and Nassira Gaouar-Benyelles

Reduced intracellular drug accumulation augments fluoroquinolone and β-lactam drugs resistance in clinical Gram negative bacteria from Nigeria
D. Olusoga Ogbolu, O. A. Daini, A. O. Terry Alli and M. A. Webber

Variability in *Fusarium oxysporum* f.sp. *ciceris* causing wilt in chickpea
Amandeep Kaur, Vineet K. Sharma, Asmita Sirari, Jaspal Kaur, Gursahib Singh and Pardeep Kumar

Exploiting novel rhizosphere *Bacillus* species to suppress the root rot and wilt pathogens of chickpea
Kodoth Padinhare Smitha, Rajeswari Mohan, Alice Devadason and Thiruvengadam Raguchander

Different dosages of SALMEX® to control *Clostridium perfringens* in poultry feed ingredients
Mariana Fröner Casagrande, Marita Vedovelli Cardozo, Livia Boarini, Mariana Casteleti Beraldo-Massoli, Flávio Alves Longo, Juliano Vittori and Rubén Pablo Schocken-Iturrino

Bacterial inoculation effect on soil biological properties, growth, grain yield, total phenolic and flavonoids contents of common buckwheat (*Fagopyrum esculentum* Moench) under hilly ecosystems of North-East India
Raghavendra Singh, Subhash Babu, R. K. Avasthe, G. S. Yadav, Tirtha Kumari Chettri, C. D. Phempunadi and Tarama Chatterjee

academicJournals

Vol. 9(15), pp. 1060-1074, 15 April, 2015
DOI: 10.5897/AJMR2014.7313
Article Number: 4B368F652639
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Arbuscular mycorrhizal fungi in the *Jatropha curcas* rhizosphere

Bruno Coutinho Moreira[1], Ana Lúcia Rodrigues[1], Sabrina Feliciano Oliveira[1], Paulo Sérgio Balbino Miguel[1], Denise Mara Soares Bazzolli[1], Sidney Luiz Stürmer[2] and Maria Catarina Megumi Kasuya[1]*

[1]Departamento de Microbiologia, Universidade Federal de Viçosa, Viçosa, Minas Gerais, 36570-000, Brazil.
[2]Departamento de Ciências Naturais, Universidade Regional de Blumenau, Blumenau, Santa Catarina, 89012-900, Brazil.

Received 1 December, 2014; Accepted 13 April, 2015

***Jatropha curcas* L. is a Euphorbiaceae characterized as drought tolerant, with low nutrient exigency and resistant to pests and diseases; furthermore, its seeds have a high content of good quality oil, which makes it a potential plant species for biodiesel production. The association of *J. curcas* with arbuscular mycorrhizal fungi (AMF) may enhance some of these promising features. The aim of this work was to assess AMF community associated with different genotypes of *J. curcas* (different accessions of a germoplasm bank) grown in the same area and in plants of the same genotype grown in different regions to identify promising fungi in this association. The AMF community was assessed by morphological analysis and by polymerase chain reaction-denaturing gradient gel electrophoresis fingerprinting approach (PCR-DGGE) and sequencing of 18S rDNA. Twenty-seven species of AMF were identified morphologically, in addition to five additional ones identified by sequencing of DGGE bands. In both analyses, some genera and species were found in common, including *Glomus* and *Acaulospora*. In the same accession or in samples obtained from the same genetic material, but cultivated in neighboring regions, the AMF community had a greater similarity, showing a possible influence of the genetic material and of climatic conditions on the AMF community. Regardless of the AMF community, these plants present a high percentage of mycorrhizal colonization and a relatively high number of AMF spores, suggesting an important relationship with mycorrhizal association.**

Key words: Nested PCR-DGGE, *Jatropha curcas,* arbuscular mycorrhizal fungi (AMF) community, Glomus, spore morphology.

INTRODUCTION

The necessity to reduce the use of fossil fuels has intensified research to develop technologies for renewable energy (Sharma and Singh, 2009). As an alternative, the production of biofuels has been increased

*Corresponding author. E-mail: mkasuya@ufv.br.

worldwide. As a substitute of petrodiesel, biodiesel must be technically feasible, economically competitive and environmentally sustainable (Demirbas, 2007).

Within this context, *Jatropha curcas* L. is an important plant due to seed quality (Behera et al., 2010). *J. curcas* (Euphorbiaceae), known as the physic nut, is native of tropical America and has been broadly dispersed through the tropical and subtropical areas of Africa and Asia (Schmook and Serralta-Peraza, 1997; Openshaw, 2000). It is a perennial shrub, with 5 to 7 m height (Achten et al., 2008; Drumond et al., 2009), and an average lifespan of 50 years (Achten et al., 2008). Besides having a high content of good quality oil in their seeds, the plant is considered drought tolerant and able to grow in soils with low nutrient contents, requires little manual labor for cultivation, does not compete with other cash crops and tolerates well pests and diseases (Openshaw, 2000; Jongschaap et al., 2007; Achten et al., 2008; Behera et al., 2010).

The beneficial association between *J. curcas* and arbuscular mycorrhizal fungi (AMF) has been demonstrated (Openshaw, 2000; Achten et al., 2008; Charoenpakdee et al., 2010). This association occurs between some soil fungi and most terrestrial plants, is present in nature more than 400 million years and is found in approximately 80% of plants, including most of agricultural, horticultural and forestry species (Pozo and Azcón-Aguilar, 2007). Plants that participate in mycorrhizal symbioses have an increasing nutrient uptake (Smith et al., 2010), a higher tolerance to drought and salt stresses than non-mycorrhizal plants (Augé, 2001), and a greater resistance to the effects of heavy metals (Rozpadek et al., 2014). Besides increase, the resistance to pathogens and act as plant growth promoters (Pozo et al., 2002; Hernández-Montiel et al., 2013). Considering that environmental factors, such as soil moisture (Helgason and Fitter, 2005; Silva et al., 2014), pH, rainfall and soil type (Hazard et al., 2013) can affect AMF community and there are only a few studies emphasizing the diversity of AMF associated with physic nut under distinct edaphoclimatic conditions, identification of the common species in the rhizosphere of *J. curcas* is important, which information can be used in crop management in the field, or even in the production of mycorrhizal seedlings, in order to fully exploit the potential of this association taking into account the characteristics of each locality.

Identification of AMF species has relied mainly on the analysis of spore morphological characteristics such as color, shape and size as well as spore-wall properties (Morton, 1988; Schenck and Perez, 1990). However, changes in the spore wall, resulting from interactions with the environment and differential sporulation patterns between species of AMF, make it difficult to identify field-collected spores (Rodríguez-Echeverría and Freitas, 2006), especially in cases where microbial activity is high. In addition, an evaluation solely based in the morphology of spores provides an incomplete interpretation of the community structure of these fungi in the environment (Ma et al., 2005; Hempel et al., 2007).

The use of molecular tools to assess AMF species diversity under field conditions has allowed to detect species with low sporulation rates in soil which would have a greater difficulty to be detected by morphological analysis. In addition, molecular approaches do not usually require steps associated with the cultivation and production of fungi spores in trap cultures (Kowalchuk et al., 2002). In this context, denaturing gradient gel electrophoresis fingerprinting approach (DGGE) has been used to analyze the AMF community, allowing access to these fungi in the root systems of plants, in soil samples or even through a spore bank (Kowalchuk et al., 2002).

The aims of this study were to analyze the richness of the AMF species associated with distinct genotypes of *J. curcas* and to compare the diversity of these fungi in different soil and climatic conditions using the classical method of identification (morphology), complemented with molecular assessments.

MATERIALS AND METHODS

Sampling

Samples of soil and root system of *J. curcas* were collected at the germplasm bank of experimental station of Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG), Unidade Regional Norte de Minas (URENM) in Nova Porteirinha, and on commercial plantations in Viçosa and Canaã, in the state of Minas Gerais, Brazil, between the months of February and April 2010. In all areas, there were only *J. curcas* plants as monocrop, so the AMF community was not influenced by any other plant species. The soils at EPAMIG are sandy loam in texture with average rainfall annual of 876 mm, whereas those at the commercial plantation are sandy clay with average rainfall annual of 1,221.4 mm (Table 1).

At the EPAMIG experimental station, 44 accessions of *J. curcas* were originally obtained from five different regions (MA-Matinha; PA-Paciência; BA-Banavit; BR-Barbosa and SE-Sub-estação Janaúba), and three plants per accession were sampled. Due to the limited number of plants in Canaã and Viçosa, six and nine plants, respectively, were selected at random.

The soil samples were collected using a cylindrical ring of 5-cm diameter and 20-cm depth to obtain a standardized volume of the samples. Two samples of the soil and root system per plant under the canopy were collected to obtain a composite sample.

Samples of the root system were collected manually at the same points of soil sampling for evaluating the percentage of roots with mycorrhizal colonization.

Morphological characterization of AMF

AMF spores were extracted from a 100 cm^3 aliquot of each soil sample using the wet-sieving technique (Gerdemann and Nicholson, 1963), followed by centrifugation in water and then in a 45% sucrose solution. Subsequently, the quantification and separation of spores were performed under a dissecting microscope using morphological characteristics (shape, color and

Table 1. Physical and chemical characteristics of soils collected from the areas of Viçosa, Canaã e Nova porteitinha.

Area	pH	P	K	Ca^{+2}	Mg^{+2}	Al^{+3}	H+Al	SB	$CEC_{(t)}$
	H_2O	----mg/dm^3----		----------cmolc/dm^3----------					
Canaã/MG	4.40	45.70	65.00	3.00	0.40	0.40	5.78	3.57	3.97
Nova Porteirinha/MG	6.65	30.00	290.00	3.10	1.80	0	1.49	5.64	5.64
Viçosa/MG	5.20	19.60	158.00	4.00	1.20	0.10	2.81	5.60	5.70
	$CEC_{(T)}$	V	m	OM	P-rem	Clay	Silt	Sand	
	cmolc/dm^3	---------%---------		dag/Kg	mg/L	----------%----------			
Canaã/MG	9.35	38.00	10.00	3.70	33.20	36	10	54	
Nova Porteirinha/MG	7.13	79.00	0	0.80	50.9	12	22	66	
Viçosa/MG	8.41	67.00	2.00	2.90	29.60	33	17	50	

SB, sum of bases; $CEC_{(t)}$, effective cationic exchange capacity; $CEC_{(T)}$, cationic exchange capacity in pH 7,0; V(%), base saturation; m(%), Al saturation; OM, organic matter; P-rem, remaining phosphorus.

size).

For AMF species identification, the spores were separated according to their morphotypes and mounted on slides with pure polyvinyl-lacto-glycerol (PVLG) and in PVLG mixed with Melzer (1:1 v:v). Identification was made using the descriptions of reference cultures from the International Culture Collection of Vesicular-arbuscular and Arbuscular Mycorrhizal Fungi (INVAM, 2010) (at http://invam.caf.wvu.edu) and by consulting the protocols available at the AMF-phylogeny website (www.lrz.de/~schuessler/amphylo). The genera and families presented in this paper follow the consensus classification of Redecker et al. (2013).

Root colonization

For evaluation of mycorrhizal colonization, roots were kept in FAA (formalin: Alcohol-ethanol: acetic acid, 0.5:9:0.5) and stored for later analysis. The roots were subjected to bleaching in a solution of KOH 10 % (w:v) for 12 h, washed in water and subsequently immersed in HCl 1 % (v:v) for 5 min, followed by staining in 0.05 % trypan blue in lactoglycerol (w:v) at 70°C for 40-60 min (Phillips and Hayman, 1970). Root colonization was quantified by using the gridline-intersect method (Giovannetti and Mosse, 1980).

Soil DNA extraction and reference AMF species

Analysis by denaturing gradient gel electrophoresis (DGGE) was performed using DNA fragments corresponding to the 18S rDNA genes from AMF, as described by Liang et al. (2008), with modifications.

Approximately 10 g of soil sample for each treatment was crushed with the aid of the mortar and pestle to break the aggregates. From these samples, only 1 g was used for the extraction of the total DNA using an *UltraClean™ Soil DNA Isolation* kit (*MO BIO Laboratories, Solana Beach, CA, USA*), according to the manufacturer's recommendations.

The total DNA of reference AMF species was extracted using the same kit, but the AMF were concentrated from 50 cm^3 using a wet-sieving technique (Gerdemann and Nicholson, 1963) or using all the spores present in a Petri dish of an *in vitro* culture of the fungus *Rhizophagus clarus* (= *Glomus clarum*) to concentrate the spores and to ensure sufficient amounts of DNA for use as parameters in subsequent studies.

The reference markers were: a strain of *R. clarus* from *in vitro* collection (Laboratory of Mycorrhizal Associations, Universidade Federal de Viçosa -Viçosa, Brazil), *Acaulospora koskei* SCT406A, *Acaulospora tuberculata* SCT250B, *Gigaspora albida* PRN201A, *Gigaspora decipiens* SCT304A and *Dentiscutata heterogama* (= *Scutellospora heterogama*) PNB102A. Fungal isolates were obtained from the International Culture Collection of Glomeromycota (CICG - www.furb.br/cicg, at Universidade Regional de Blumenau, Blumenau, Brazil).

These reference markers were used to verify the pattern of bands in the DGGE gel of isolated AMF species, to verify the correlation of these species by morphological and molecular characterization, and besides being used as a reference in the DGGE gels.

Nested-PCR strategy for amplification of 18S rDNA fragments

The primers used in the first round for amplification of the 18S rDNA were AM1 (5'-GTTTCCCGTAAGGCGCCGAA-3') (Helgason et al., 1998) in combination with the universal primer for eukaryotes, NS31 (5'-TTGGAGGGCAAGTCTGGTGCC-3') (Simon et al., 1992).

Polymerase chain reactions (PCR) were performed in thin-walled PCR tubes, 0.5 mL, using the enzyme Go Taq DNA Polymerase Flex® (Promega, Madison, USA) in a volume of 50 µL according to the manufacturer's recommendations. Negative controls consisted of Milli-Q water and replacing the DNA sample to confirm the results. All material used in the preparation of the reactions was previously sterilized and nuclease free.

The DNA template used for amplification of the desired region consisted of 5 µL of the DNA extracted from the AMF spores, which were used as a reference, and DNA extracted from the soil. The reaction mixture for performing the PCR was composed of 200 µmol L^{-1} each deoxynucleoside triphosphates, 1.5 µmol L^{-1}, $MgCl_2$, 0.2 µmol L^{-1} of each primer and 1.25 U GoTaq DNA polymerase Flex®. Acetylated bovine serum albumin (BSA, Promega) was also added to each reaction to potentiate the action of polymerase (0.8 µg µL^{-1}). The PCR amplifications were performed in a thermocycler (Mastercycler epgradient, Eppendorf) using the following steps: a first cycle of 1 min at 94 °C, 1 min at 66 °C and 1 min 30 s at 72 °C, followed by an additional 30 cycles of 30 s at 94 °C, 1 min at 66 °C and 1 min 30 s at 72 °C and finally a 10 min final extension at 72 °C. To confirm the presence of the amplified product, aliquots of of 5 µL of the products of PCR reactions were submitted to electrophoresis on agarose gel 0.8 % (w:v) stained with ethidium bromide (0.5 µg mL^{-1}) and visualized under UV light photodocumentation

imaging system (Loccus Biotecnologic L-Pix Chemi).

The amplicon corresponding to the first PCR reaction resulted in DNA fragments of approximately 560 bp. To obtain a smaller DNA fragment for carrying out the DGGE technique a second round of PCR reactions was performed (Nested-PCR).

The product of the first PCR reaction was diluted 10 times in sterile Milli-Q water and 1 µL containing about 25 ng μL^{-1} of the DNA used as template. We used the primers NS31-GC (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGTTGGAGGGCAAGTCTGGTGCC-3') (Kowalchuk et al., 2002) and Glo1 (5'-GCCTGCTTTAAACACTCTA-3') (Cornejo et al., 2004), employing the same reaction mixture as in the first round of PCR. An initial denaturation of 5 min at 94°C was performed, followed by 35 cycles of 45 s at 94°C, 45 s at 52°C, 1 min at 72°C and the extension end of fragments at 72°C for 30 min. To confirm the presence of the product, an aliquot of 5 µL of PCR was verified by electrophoresis on agarose gel 1.5 % (w:v) stained with ethidium bromide (0.5 µg mL^{-1}) and visualized under UV light photodocumentation imaging system (Loccus Biotecnologic L-Pix Chemi).

Analysis of the PCR products by DGGE

From the products obtained by the nested-PCR technique using the primers, Glo1 and NS31-GC, approximately 250 ng of DNA from each samples were analyzed by DGGE (Modelo *Dcode™ System* – BIO-Rad California, USA).

The references were performed as described for the field samples and approximately 300 ng of the DNA mixture of these species were used as marker for the analysis of DGGE.

The polyacrylamide gel used contained 8% (w:v) acrylamide:bisacrylamide (37.5:1) in Tris-acetate-EDTA (TAE) 1X (Tris/acetic acid/EDTA, pH 8.0). A linear denaturing gradient was formed with the aid of the trainer Hoefer gradient SG50 (Amersham Biosciences) and the mixture of two stock solutions of polyacrylamide, to obtain a final gradient ranging from 36 to 50% that was used for all analysis, where the condition of 100% of the denaturing agents consisted of urea 7 mol L^{-1} (Sigma, Cat # U5378) and 40 % formamide (v:v) (Sigma, Cat # F9037) and another solution was created without these compounds.

All the DGGE analysis were performed in 1X TAE buffer at a constant temperature of 60°C at 80 V for a period of 10 min, followed by 60 V for 20 h. The gels had a thickness of 0.75 mm and dimensions of 16 x 16 cm and were stained, after completion of electrophoresis, for 30-40 min in solution of 1X SYBR Gold® (Sigma-Aldrich) according to the manufacturer's recommendations. The images of the gels were observed under UV light and were then captured and digitized using a photodocumentation imaging system (Loccus Biotecnologic L-Pix Chemi).

Selection and DNA fragment sequencing

Based on the different profiles obtained by DGGE, the bands showing greater intensity in each area were selected (Figure 3). The relative intensities of the bands were considered to be the frequency, which these species occur and their DNA fragments were collected with the aid of sterile tips, and were transferred to 0.5 mL microtubes containing 30 µL of sterile Milli-Q water for reamplification using PCR.

The new PCR reaction was performed in an identical manner to that used in the nested-PCR, using of the primers NS31 and Glo1. The selected fragments were sequenced by Macrogen, Inc. (Korea). Subsequently, the sequences obtained were analyzed using the BLASTn tool -NCBI (Altschul et al., 1997).

Statistical analysis

Each treatment consisted of grouping three plants collected in Viçosa and Canaã and by three replicates of each accession obtained in Nova Porteirinha. They were evaluated in relation to the number of spores and the percentage of colonization. The data were subjected to an analysis of variance (ANOVA) at α level of 5%. The means were compared using a Tukey test ($P \leq 0.10$). The data relating to the spore counts were previously via normalized log (x+1) and mycorrhizal colonization via an arcsin$\sqrt{(x/100)}$ transformation for a subsequent ANOVA. Considering the large number of samples collected in Nova Porteirinha, the frequency (Freq) of each species found in the area was calculated according to the following formula: Freq = (number of accessions where the AMF species was found / total number of accessions)*100.

To analyze the profile of AMF in these soils and generate the dendrogram representing the distance and pattern of bands corresponding to the 18S rDNA gene of AMF, the images of the obtained gels were analyzed and aligned based on the external markers with the reference species by BioNumerics version 6.0 (Applied Maths, Inc., Austin, Texas, USA).

RESULTS

There were no differences ($P \leq 0.10$) in mycorrhizal colonization between samples of the *J. curcas* root collected in Viçosa and Canaã, as well as among the accessions collected in Nova Porteirinha. However, Canaã region presented a higher number of spores ($P \leq 0.10$) per 100 cm^3 of soil (Figure 1). All root samples analyzed showed typical structures of AMF colonization, with hyphae, arbuscules and/or vesicles; morphologically distinct AMF spores were observed in all soils.

Diversity of AMF by morphological characteristics

A total of 27 morphospecies of AMF were detected in all areas, belonging to nine genera and seven families in the Glomeromycota. Twelve species were identified only at the genus level and most of them formed glomoid spores and assigned conservatively to the genus *Glomus*. The largest number of species was recovered from Nova Porteirinha (21) where the largest number of samples was obtained, followed by Viçosa (14) and Canaã (7).

Glomus was the most common genera recovered (12 species) followed by *Acaulospora* (8 species). Other genera were represented by one species each (Table 2). In Nova Porteirinha, *Acaulospora morrowiae* was the most frequent species (93 % frequency), followed by *A. mellea* (74 %), *Glomus* sp. (72 %), *Pacispora* sp. (72 %) and *R. diaphanum* (= *Glomus diaphanum)* (69%). The remaining species were found in less than 50% of the samples (Table 2).

Identification of AMF by molecular tools

DNA extraction from soils and referencing the AMF spores as markers was performed successfully. After diluting

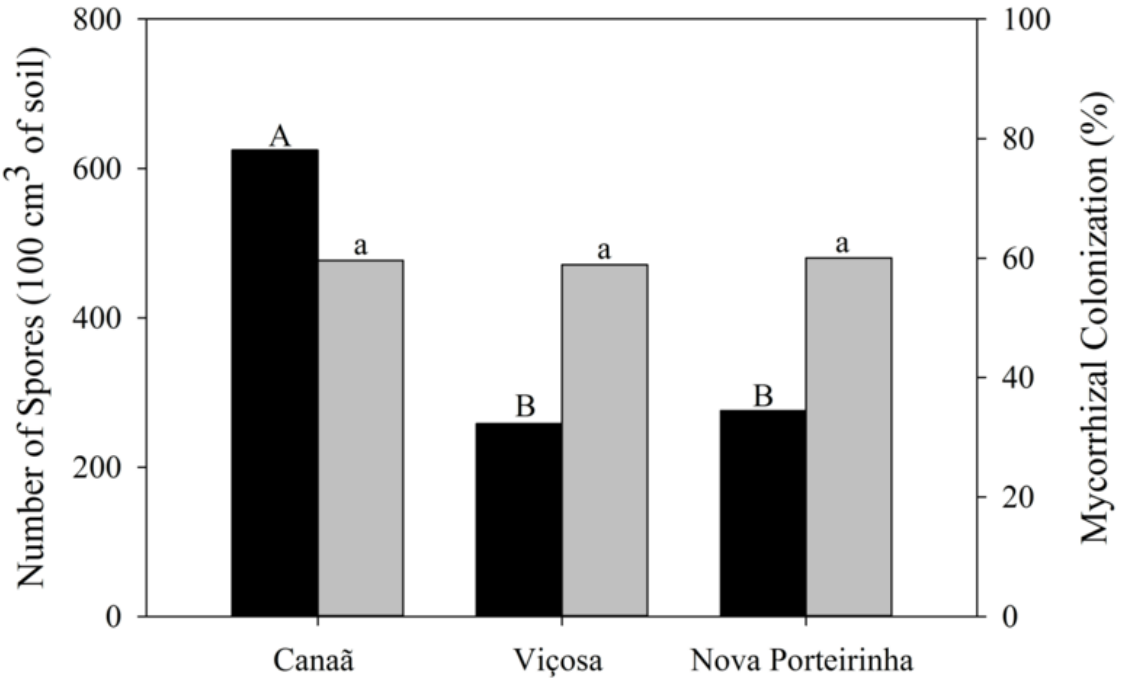

Figure 1. Number of spores of Arbuscular Mycorrhizal Fungi (■) (per 100 cm^3) and mycorrhizal colonization (■) in the rhizosphere of *J. curcas* in Canaã, Viçosa and Nova Porteirinha. Means followed by the same uppercase letter do not differ from each other Tukey test (P≤ 0.10) for number of spores and means followed by the same lowercase letter do not differ from each other by Tukey test (P≤ 0.10) for mycorrhizal colonization (%).

the products of this first round of amplification and its subsequent amplification with primers Glo1/NS31-GC (nested-PCR), we obtained amplicons of the expected size (approximately 230 bp) in all samples, as also observed by Cornejo et al. (2004) and Liang et al. (2008).

The amplicons obtained by nested-PCR generated a profile of several bands in the DGGE gel, characteristic for each reference species analyzed (Figure 2).

Some predominant bands in the profiles of the species used as references were eluted and sequenced. The obtained sequences were analyzed using the BLASTn tool (NCBI) that showed the same species identified by morphological techniques or at least as belonging to the same family (Table 3). All species used as reference markers species that were identified by morphological characteristics were confirmed by molecular analysis.

The nested-PCR, using the primer pair Glo1/NS31-GC, resulted in DNA fragments corresponding to the partial 18S rDNA sequence in all analyzed samples. The profiles of separation of fragments in these bands of DGGE gels are shown in Figure 3.

Difference on distribution patterns of bands were observed in samples between regions. Band positions from Viçosa and Canaã samples showed a more pronounced difference between replicates. Although some variables interfere with the molecular analysis of soil microbial communities, it is possible to make a comparison between the compositions of communities of microorganisms in the areas under study using BioNumerics software (Figure 3).

Similarity between AMF communities was larger between accessions of the same region. Likewise, the samples from Canaã 01 and 02 and Viçosa 01 and 03 formed a cluster, indicating that genetic material of the same origin occurring in fairly remote regions, with similar climatic conditions, results in similar AMF communities in rhizosphere of *J. curcas*.

Sequencing of selected DGGE bands and identification of AMF

From the sequence analysis performed by the BLASTn tool (NCBI) identity values ranged from 81-100% (Table S1).

Five species of four genera, beyond those already identified by morphological characteristics were identified after sequencing of the 18S rDNA gene fragments: *Gigaspora decipiens* Hall and Abbott, *Gigaspora gigantea* (Nicol. and Gerd.) Gerd. and Trappe, *R. clarus* Nicol. and Schenck, *Scutellospora dipapillosa* (Koske and Walker) Walker and Sanders and *Dentiscutata heterogama*

Table 2. Arbuscular Mycorrhizal Fungi (AMF) occurring in rhizosphere of *J. curcas* in Viçosa (V), Canaã (C) and Nova Porteirinha (NP) and the frequency of AMF occurrence in Nova Porteirinha.

AMF species	V	C	NP	Freq NP* (%)
Family Acaulosporaceae				
Acaulospora delicata Walker, Pfeiffer and Bloss	+	-	+	18.6
Acaulospora excavata Ingleby and Walker	-	-	+	9.3
Acaulospora mellea Spain and Schenck	-	+	+	74.4
Acaulospora morrowiae Spain and Schenck	-	-	+	93.0
Acaulospora paulinae Blaszkowski	+	-	-	-
Acaulospora scrobiculata Trappe	+	-	+	25.6
Acaulospora walkeri Kramadibrata and Hedger	-	-	+	9.3
Acaulospora sp.	-	-	+	4.6
Family Archaeosporaceae				
Archaeospora trappei (Ames and Linderman) Morton and Redecker	-	-	+	2.3
Family Claroideoglomeraceae				
Claroideoglomus etunicatus (Becker and Gerdemann)	+	-	+	2.3
Family Glomeraceae				
Rhizophagus diaphanum (Morton and Walker) Schussler and Walker	+	+	+	69.8
Funneliformis mosseae (Nicol. and Gerd.) Schussler and Walker	+	-	+	4.6
Glomus viscosum (Nicol.)	+	-	-	-
Glomus sp	-	-	+	2.3
Glomus sp 1	+	-	-	-
Glomus sp 2	+	+	+	72.1
Glomus sp 3	+	+	-	-
Glomus sp 4	+	-	-	-
Glomus sp 5	+	+	-	-
Glomus sp 6	-	+	+	7
Glomus sp 7	-	+	+	2.3

(+) presence or (-) absence of the species in the area. * Freq = (number of accessions where the AMF species were found / total number of accessions)*100.

(Nicol. and Gerdemann) Sieverding, Souza and Oehl.

These species were not recovered previously as spores and therefore increased AMF diversity associated with *J. curcas* to 32 species.The bands eluted in the same position in the gel (Figure 3), collected from the different accessions of *J. curcas* in Nova Porteirinha, generally indicated that the AMF species were phylogenetically close to each other, especially at the genus level (Table S1).

DISCUSSION

Assessment of AMF diversity based on field-collected spores indicated the dominance of the family Glomeraceae, represented in this study by *Glomus, Rhizophagus* and *Funneliformis,* while molecular analysis revealed the prevalence of members of the Gigasporaceae (*Gigaspora, Scutellospora* and *Dentiscutata*) (Table S1). Similar results were found by Alguacil et al. (2012) who found species of *Glomus* being predominantely associated with *J. curcas*.

The predominance of small size *Glomus* spores may be linked to survival and propagation strategies found in this genus (Liang et al., 2008). The largest number of *Glomus* species in all three areas of study may be related to the high adaptability of this genus to the variations of temperature and soils, besides its ability to survive in a pH ranging from acidic to alkaline (Ho, 1987) and adapting to the disturbances in the soil (Oehl et al., 2010).

We also detected members of *Acaulospora*, *Archaeospora*, *Pacispora* and *Paraglomus* in rhizosphere of this plant. *Acaulospora* and *Glomus* have been reported as the most frequently found genera associated with *J. curca,* with 16 and 10 species, respectively (Charoenpakdee et al., 2010). Furthermore, these authors also found *Entrophospora* (1 species), *Gigaspora* (2 species) and *Scutellospora* (5 species). In the study by Charoenpakdee et al. (2010) as our, the species

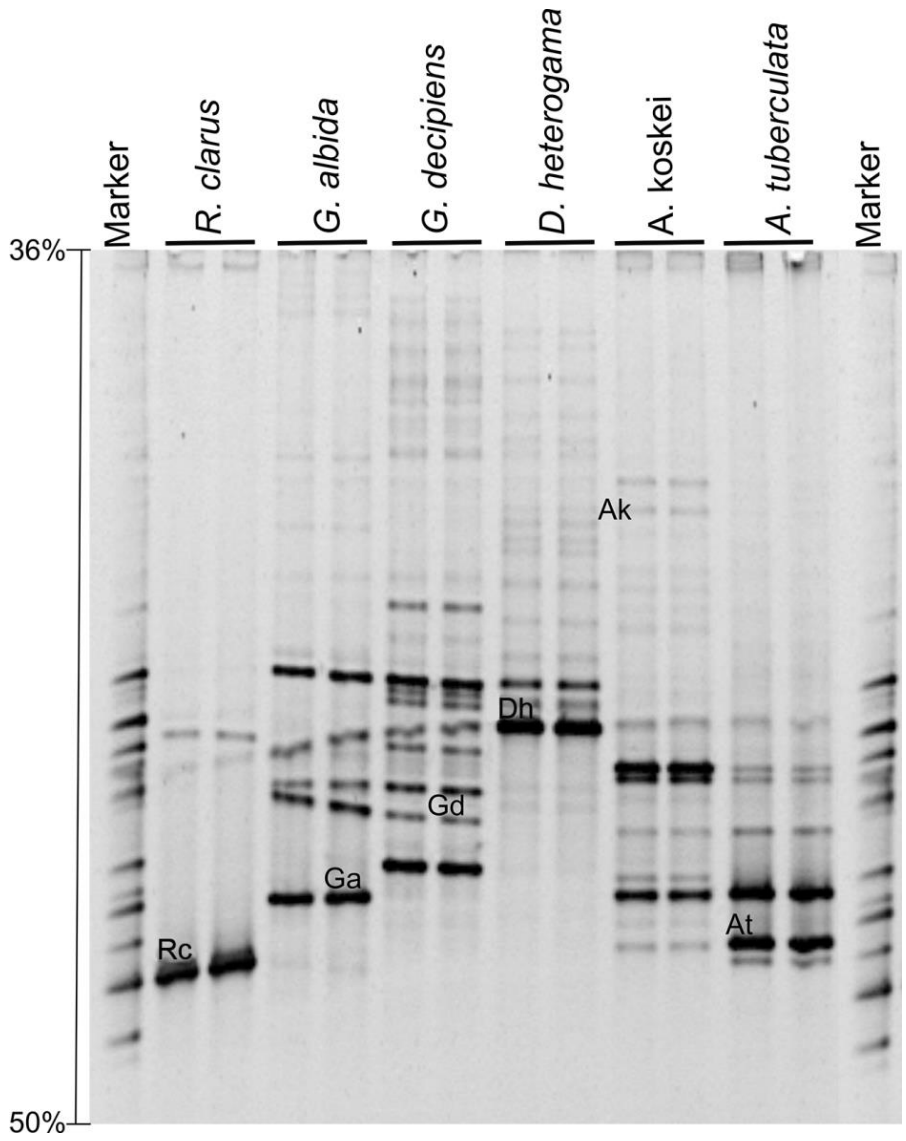

Figure 2. Profile of the bands corresponding to the 18S rDNA fragments of AMF species used as markers obtained by DGGE.

Table 3. Identity of the bands selected and eluted from the DGGE gel of Arbuscular Mycorrhizal Fungi used as reference markers.

Position of the gel band	Closest match from GenBank (% sequence similarity by BLASTn)	Genbank accession no.
Rc	*Rhizophagus clarus* (99%)	AJ852597.1
Ga	*Gigaspora* sp (98%)	EF447242.1
Gd	*Gigaspora decipiens* (100%)	AY641812.1
Dh	*Dentiscutata heterogama* (100%)	NG_017177.1
Ak	*Acaulospora* sp (96%)	AY919854.1
At	*Acaulosporaceae* (98%)	GU198548.1

The codes Rc, Ga, Gd, Dh, Ak, At, indicate the bands eluted and sequenced in DGGE gel shown in Figure 2. Only a few sequenced bands are shown in this table."

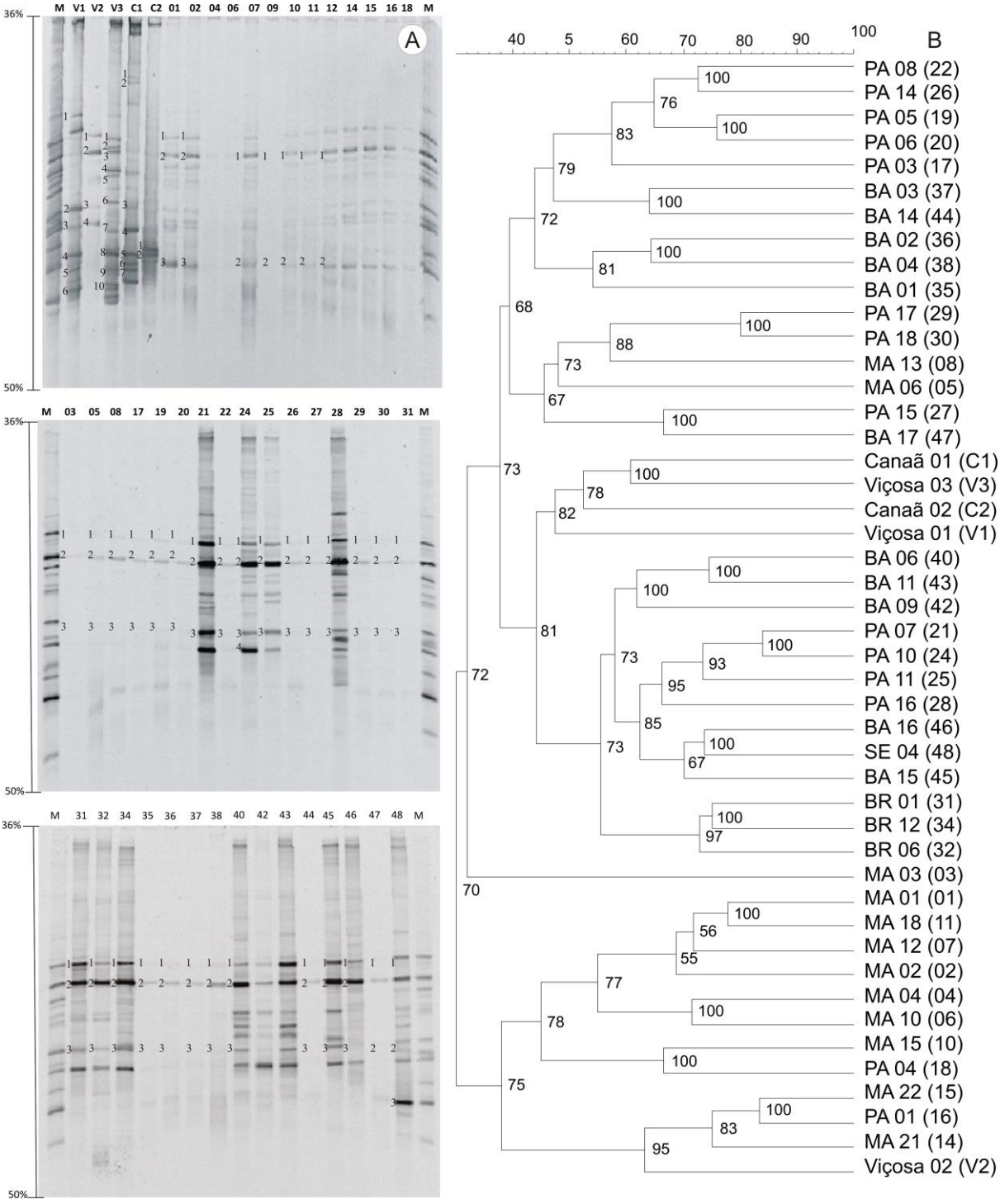

Figure 3. DGGE profile of AMF 18S rDNA fragments from Viçosa, Canaã and Nova Porteirinha. **(A)** The denaturant gradient increases from 36% on the top to 50%. M, Markers. Samples were collected in (V) Viçosa, (C) Canaã and (01 to 48) identification of each accession was collected in Nova Porteirinha/MG. The bands numbered indicate the ones which were eluted, amplified in PCR, sequenced and analyzed by BLASTn. **(B)** The UPGMA tree inferred from AMF 18S rDNA fragments from DGGE gels. The accessions of *J. curcas* deposited in the bank germplasm originally obtained from five different regions are identified by the abbreviations: MA, Matinha; PA, Paciência; BA, Banavit; BR, Barbosa and SE, Sub-estação Janaúba. The numbers indicate cophenetic correlations, which are estimates of the faithfulness of each subcluster of the dendrogram.

Table S1. Identity of the bands selected and elutes from the DGGE gel of Arbuscular Mycorrhizal Fungi samples obtained in Viçosa (V), Canaã (C) and for each accession in Nova Porteirinha (Ac).

Position the gel band	Closest match from GenBank (% sequence similarity by BLASTn)	Genbank accession no.
V1-1	Uncultured *Glomus* clone NES17#G16 18S ribosomal RNA gene, partial sequence (94 %)	GU353935.1
V1-2	Uncultured *Glomus* partial 18S rRNA gene, clone 30_14.S-NT (95 %)	AM412085.1
V1-3	Uncultured *Glomus* clone HDALG14 18S ribosomal RNA gene, partial sequence (98 %)	GQ336527.1
V1-4	Uncultured *Glomus* clone HDALG14 18S ribosomal RNA gene, partial sequence (97 %)	GQ336527.1
V1-5	*Glomus* sp. CH3263078 partial 18S rRNA gene, isolate CH3263078 (94 %)	FR690122.1
V1-6	*Glomus* sp. CH3263078 partial 18S rRNA gene, isolate CH3263078 (96 %)	FR690122.1
V2-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (96 %)	AJ852609.1
V2-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (99 %)	AJ852609.1
V2-3	*Gigaspora decipiens* isolate DGGE band AU102-5 18S ribosomal RNA gene, partial sequence (100 %)	AY641812.1
V2-4	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (100 %)	EF447242.1
V3-1	Uncultured *Glomus* isolate DGGE band 123 14.c2.1.1.14c2 18S ribosomal RNA gene, partial sequence (97 %)	HQ323622.1
V3-2	Uncultured *Glomus* clone T22L1SP 18S small subunit ribosomal RNA gene, partial sequence (97 %)	EF177648.1
V3-3	Uncultured *Glomus* clone T22L1SP 18S small subunit ribosomal RNA gene, partial sequence (94 %)	EF177648.1
V3-4	Uncultured *Glomus* clone K179c6 18S ribosomal RNA gene, partial sequence (95 %)	DQ336464.1
V3-5	Uncultured *Gigasporaceae* clone FVDWSEP01EB9KY 18S ribosomal RNA gene, partial sequence (92 %)	GU198545.1
V3-6	Uncultured *Glomus* partial 18S rRNA gene, isolate PS41G (81 %)	FM955850.1
V3-7	Uncultured *Glomus* clone G1C4A1Z 18S small subunit ribosomal RNA gene, partial sequence (92 %)	EF177562.1
V3-8	Uncultured *Glomus* clone G10_2L2SP 18S small subunit ribosomal RNA gene, partial sequence (93 %)	EF177547.1
V3-9	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (94 %)	AJ852609.1
V3-10	Uncultured *Glomus* clone HDAMG10 18S ribosomal RNA gene, partial sequence (89 %)	GQ340787.1
C1-1	Uncultured *Glomus* clone DNA62_3 18S ribosomal RNA gene, partial sequence (96 %)	HM440265.1
C1-2	_	_
C1-3	Uncultured *Glomus* clone K230c5 18S ribosomal RNA gene, partial sequence (89 %)	DQ336521.1
C1-4	Uncultured *Glomus* partial 18S rRNA gene, isolate PS41G (94 %)	FM955850.1
C1-5	Uncultured *Gigasporaceae* clone FVDWSEP01CE9TJ 18S ribosomal RNA gene, partial sequence (87 %)	GU198546.1
C1-6	_	_
C1-7	Uncultured *Glomus* clone 14 group 5 18S small subunit ribosomal RNA gene, partial sequence (93 %)	EF109875.1
C2-1	Uncultured *Gigasporaceae* clone FVDWSEP01EB9KY 18S ribosomal RNA gene, partial sequence (91 %)	GU198545.1
C2-2	Uncultured *Glomus* small subunit ribosomal RNA gene, partial sequence (97 %)	DQ371697.1
Ac- 01-1	*Gigaspora gigantea* partial 18S rRNA gene, clone G-5 (90 %)	AM746154.1
Ac- 01-2	*Scutellospora heterogama* partial 18S rRNA gene, clone pWD163-2-6 (92 %)	AJ306434.1
Ac- 01-3	Uncultured *Glomus* clone NES01#D16 18S ribosomal RNA gene, partial sequence (90 %)	GU353768.1
Ac- 02-1	_	_
Ac- 02-2	*Scutellospora heterogama* strain INVAM FL225 18S ribosomal RNA, partial sequence (98 %)	NG_017177.1

Table S1.Contd.

Position the gel band	Closest match from GenBank (% sequence similarity by BLASTn)	Genbank accession no.
Ac- 02-3	Uncultured *Glomus* partial 18S rRNA gene, isolate PS41G (82 %)	FM955850.1
Ac- 03-1	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (98 %)	EF447242.1
Ac- 03-2	*Scutellospora heterogama* strain INVAM FL225 18S ribosomal RNA, partial sequence (99 %)	NG_017177.1
Ac- 03-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (98 %)	EF447242.1
Ac- 05-1	*Scutellospora heterogama* strain INVAM FL225 18S ribosomal RNA, partial sequence (99 %)	NG_017177.1
Ac- 05-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (100 %)	AJ852609.1
Ac- 05-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (97 %)	EF447242.1
Ac- 07-1	_	_
Ac- 07-2	_	_
Ac- 08-1	Uncultured *Gigasporaceae* clone LES13#I21 18S ribosomal RNA gene, partial sequence (96 %)	GU353712.1
Ac- 08-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (100 %)	AJ852609.1
Ac- 08-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (99 %)	EF447242.1
Ac- 09-1	*Scutellospora dipapillosa* rDNA for small subunit rRNA (87 %)	Z14013.1
Ac- 09-2	_	_
Ac- 10-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (94 %)	AJ852609.1
Ac- 10-2	Uncultured *Glomus* clone FVDWSEP01EPG8A 18S ribosomal RNA gene, partial sequence (85 %)	GU198598.1
Ac- 11-1	Uncultured *Glomus* clone NES34#D30 18S ribosomal RNA gene, partial sequence (96 %)	GU353956.1
Ac- 11-2	*Glomus clarum* 18S rRNA gene, isolate UFPE08 (93 %)	AJ852597.1
Ac- 12-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (99 %)	AJ852609.1
Ac- 12-2	*Glomus clarum* 18S rRNA gene, isolate UFPE08 (100 %)	AJ852597.1
Ac- 17-1	*Scutellospora heterogama* strain INVAM FL225 18S ribosomal RNA, partial sequence (99 %)	NG_017177.1
Ac- 17-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (100 %)	AJ852609.1
Ac- 17-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (97 %)	EF447242.1
Ac- 19-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (97 %)	AJ852609.1
Ac- 19-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (100 %)	AJ852609.1
Ac- 19-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (98 %)	EF447242.1
Ac- 20-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (97 %)	AJ852609.1
Ac- 20-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (100 %)	AJ852609.1
Ac- 20-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (98 %)	EF447242.1
Ac- 21-1	Uncultured *Gigasporaceae* clone LER04#P36 18S ribosomal RNA gene, partial sequence (93 %)	GU353463.1
Ac- 21-2	*Scutellospora heterogama* strain INVAM FL225 18S ribosomal RNA, partial sequence (100 %)	NG_017177.1
Ac- 21-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (96 %)	EF447242.1
Ac- 21-4	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (99 %)	EF447242.1
Ac- 22-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (98 %)	AJ852609.1
Ac- 22-2	_	_

Table S1. Contd.

Position the gel band	Closest match from GenBank (% sequence similarity by BLASTn)	Genbank accession no.
Ac- 22-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (97 %)	EF447242.1
Ac- 24-1	*Scutellospora heterogama* strain INVAM FL225 18S ribosomal RNA, partial sequence (99 %)	NG_017177.1
Ac- 24-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (95 %)	AJ852609.1
Ac- 24-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (98 %)	EF447242.1
Ac- 24-4	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (99 %)	EF447242.1
Ac- 25-1	*Scutellospora heterogama* partial 18S rRNA gene, clone pWD163-2-6 (96 %)	AJ306434.1
Ac- 25-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (99 %)	AJ852609.1
Ac- 25-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (97 %)	EF447242.1
Ac- 26-1	*Scutellospora heterogama* partial 18S rRNA gene, clone pWD163-2-6 (97 %)	AJ306434.1
Ac- 26-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (92 %)	AJ852609.1
Ac- 26-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (97 %)	EF447242.1
Ac- 27-1	*Scutellospora heterogama* strain INVAM FL225 18S ribosomal RNA, partial sequence (96 %)	NG_017177.1
Ac- 27-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (99 %)	AJ852609.1
Ac- 27-3	*Scutellospora heterogama* strain INVAM FL225 18S ribosomal RNA, partial sequence (96 %)	NG_017177.1
Ac- 28-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (98 %)	AJ852609.1
Ac- 28-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (100 %)	AJ852609.1
Ac- 28-3	*Scutellospora heterogama* strain INVAM FL225 18S ribosomal RNA, partial sequence (92 %)	NG_017177.1
Ac- 29-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (98 %)	AJ852609.1
Ac- 29-2	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (96 %)	EF447242.1
Ac- 29-3	*Scutellospora heterogama* strain INVAM FL225 18S ribosomal RNA, partial sequence (95 %)	NG_017177.1
Ac- 30-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (97 %)	AJ852609.1
Ac- 30-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (96 %)	AJ852609.1
Ac- 30-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (96 %)	EF447242.1
Ac- 31-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (93 %)	AJ852609.1
Ac- 31-2	*Scutellospora heterogama* strain INVAM FL225 18S ribosomal RNA, partial sequence (97 %)	NG_017177.1
Ac- 31-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (96 %)	EF447242.1
Ac- 32-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (96 %)	AJ852609.1
Ac- 32-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (98 %)	AJ852609.1
Ac- 32-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (97 %)	EF447242.1
Ac- 34-1	*Scutellospora heterogama* partial 18S rRNA gene, clone pWD163-2-6 (97 %)	AJ306434.1
Ac- 34-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (95 %)	AJ852609.1
Ac- 34-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (98 %)	EF447242.1
Ac- 35-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (98 %)	AJ852609.1
Ac- 35-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (93 %)	AJ852609.1
Ac- 35-3	*Scutellospora heterogama* strain INVAM FL225 18S ribosomal RNA, partial sequence (91 %)	NG_017177.1

Table S1. Contd.

Position the gel band	Closest match from GenBank (% sequence similarity by BLASTn)	Genbank accession no.
Ac- 36-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (96 %)	AJ852609.1
Ac- 36-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (97 %)	AJ852609.1
Ac- 36-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (99 %)	EF447242.1
Ac- 37-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (98 %)	AJ852609.1
Ac- 37-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (97 %)	AJ852609.1
Ac- 37-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (98 %)	EF447242.1
Ac- 38-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (98 %)	AJ852609.1
Ac- 38-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (99 %)	AJ852609.1
Ac- 38-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (98 %)	EF447242.1
Ac- 40-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (97 %)	AJ852609.1
Ac- 40-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (97 %)	AJ852609.1
Ac- 40-3	Uncultured *Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (96 %)	EF447242.1
Ac- 44-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (97 %)	AJ852609.1
Ac- 44-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (96 %)	AJ852609.1
Ac- 44-3	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (96 %)	AJ852609.1
Ac- 45-1	*Scutellospora heterogama* partial 18S rRNA gene, clone pWD163-2-6 (98 %)	AJ306434.1
Ac- 45-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (100 %)	AJ852609.1
Ac- 45-3	*Uncultured Gigasporaceae* clone FVDWSEP01EB9KY 18S ribosomal RNA gene, partial sequence (96%)	GU198545.1
Ac- 46-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (98 %)	AJ852609.1
Ac- 46-2	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (96 %)	AJ852609.1
Ac- 46-3	*Uncultured Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (94 %)	EF447242.1
Ac- 47-1	*Scutellospora heterogama* strain INVAM FL225 18S ribosomal RNA, partial sequence (98 %)	NG_017177.1
Ac- 47-2	*Uncultured Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (97 %)	EF447242.1
Ac- 48-1	*Scutellospora heterogama* 18S rRNA gene, isolate UFPE19 (97 %)	AJ852609.1
Ac- 48-2	*Uncultured Gigaspora* clone K15c2 18S ribosomal RNA gene, partial sequence (96 %)	EF447242.1
Ac- 48-3	*Glomus clarum* 18S rRNA gene, isolate UFPE08 (96 %)	AJ852597.1

The last number shows the identification of each eluted bands in each sample.

Acaulospora excavata, *Acaulospora morrowiae, Acaulospora scrobiculata, Claroideoglomus etunicatus, Cetraspora pellucida* and *Dentiscutata heterogama* were found. AMF diversity is a key factor for improving the sustainability of ecosystems, especially those with low fertility conditions (Ma et al., 2005). The high AMF species diversity found here suggest that this component of the soil biota plays a role to allow *J. curcas* grow in different habitats.

Glomeraceae is the most widespread and abundant family of AMF (Öpik et al., 2008; Öpik et al., 2010). The predominance of *Glomus* also occurs in various ecosystems, in association with various plant species as in recovered areas of Atlanti

forest in different stages of regeneration (Bonfim et al., 2013) in the rhizosphere of medicinal plants in the region of Goa, India (Radhika and Rodrigues, 2010), or associated with plants of *Agave potatorum* in semi-arid regions in Mexico (Carballar-Hernández et al., 2013), indicating that this genus seems to be more adapted to different soil conditions and ecosystems (Bonfim et al., 2013) and may become a good alternative for production of inoculum to be used even in the period of formation of *J. curcas* seedlings.

The percentages of colonization obtained in our study with averages close to 60% for all areas are close to the 54% found by Alguacil et al. (2012) and with less variation than those found by Charoenpakdee et al. (2010) (38-94%). According to these authors the presence of colonization in various conditions such as soil pH ranging from acidic to alkaline, low to moderate content of organic material, or even, high or low P availability demonstrate that this plant may present a high dependence on mycorrhizal colonization. The variation in the percentage colonization may be related to the diversity of AMF species present near the root system (Berbara et al., 2006) or compatible symbiotic plant-fungus relationship (Pouyu-Rojas et al., 2006; Porras-Soriano et al., 2009).

Although not all the bands have been sequenced, the gel profiles and the analysis of the sequences suggest that the AMF communities in the three regions analyzed, with different climatic conditions, present different characteristics and the grouping of DGGE band in the gel of samples obtained in the same regions (Figure 3) may indicate different genetic compatibility between the different accessions *J. curcas* and AMF.

The DGGE technique provides a good estimator of the community structure of these fungi in soil ecological studies (Öpik et al., 2003). The species identified by molecular analysis differed from the majority of species identified by morphological analysis except for species of *Glomus*, although some representatives have been identified at genus level, in both approaches. This difference may be attributed the reduced number of spores of some species found in the field or the dilution of spore in the sample preparation (Smith and Read, 1997).

As the band profile was generated by material collected directly from the soil rhizosphere, and there are spores of AMF forming multiple bands in the DGGE gel, the number of AMF found in the field, represented by the bands in the gel, can underestimate the community of these fungi in the area (Ma et al., 2005). Additionally, fragments of less abundant rDNA may be present in the same positions of large ones in the gel, which can occult the presence of the former and detection of possible species (Kowalchuk et al., 2002). However, the DGGE allows rapid comparisons between AMF communities from various regions and the analysis of multiple samples simultaneously, without the need for cultivation of fungi on host plants, making it a good tool for ecological studies of these microorganisms (Kowalchuk et al., 2002; De Souza et al., 2004).

Even samples obtained at each accession of *J. curcas* in Nova Porteirinha have shown differences in the presence or absence of some AMF species, previously identified by morphological characteristics, the distribution pattern of bands in the DGGE gels was very homogeneous, with presence of dominant bands occurring in the same position (Figure 3). However, it must be remarked that for morphological identification 100 cm^3 of soil was used, whereas for molecular analysis, only 1 g was used, which can be related to the lower abundance of AMF using this molecular tool.

The sequencing of bands found in different positions on the gel showed the presence of the same species, confirming the polymorphism of the 18S rDNA genes within the same AMF species (Öpik et al., 2003; Cornejo et al., 2004; Liang et al., 2008). Similar behavior was also observed by Liang et al. (2008), who worked with the following species markers: *Acaulospora scrobiculata*, *Gigaspora gigantea*, *Glomus intraradices, Funneliformis mosseae* (= *Glomus mosseae*) and *Dentiscutata heterogama*. This feature may be due the spores contain thousands of nuclei and eventually some may undergo some changes in the genes (Sanders and Croll, 2010).

One factor that may influence the analysis of the community of AMF by DGGE is the AM1 primer, which is specific to the orders Glomerales and Diversisporales and not specific to Archaeosporales and Paraglomerales (Ma et al., 2005). This contributes to the underestimation of the evaluation of diversity of AMF under field conditions. Our results partially corroborate that the members of *Paraglomus* and *Archaeospora* were detected from spores collected in the field (Table 2) but not from molecular analysis. Spores of *Acaulospora* were also found, although no molecular sequences were detected. This fact was also reported by Kowalchuk et al. (2002), which can be attributed to the selection of only some of the bands to be sequenced indicating that the use of both methods are important to obtain a more complete result of the diversity of AMF in areas of study.

Furthermore, it has been reported that this primer can amplify fragments of some ascomycetes and basidiomycetes (Helgason et al., 1998; Douhan et al., 2005). However, with the nested-PCR strategy, which combines the specificity of the partial AMF AM1 primer with the resolving power of the DGGE gel with the primer pair NS31-GC/Glo1 (Cornejo et al., 2004), it was possible to view a profile of the AMF species present in *J. curcas* rhizosphere.

Only the relative amount of AMF spores in the soil does not reflect their functional importance, that is it does not allow inferences about the intensity of mycorrhizal colonization or the importance of the distribution of hyphae in soil (Douds Jr. and Millner, 1999). However, this information allows us to carry out studies to understand

the composition of these communities of fungi and track changes due to environmental or anthropogenic disturbances.

So far, it has been reported, that some AMF species colonize *J. curcas* in some regions of Thailand, identified by the characterization of morphospecies in the rhizosphere of this plant (Charoenpakdee et al., 2010) and in Guantánamo, Cuba, by molecular analysis (Alguacil et al., 2012). This is a fundamental step towards understanding the dynamics and influence of these fungi on this plant at the field level.

Our studies revealthat in the region of Nova Porteirinha, characterized by a semi-arid climate, higher species diversity of these AMF are present in the rhizosphere of *J. curcas* compared with the regions of Viçosa and Canaã. This may be related to the greater diversity of plants present in regions with semi-arid climates with characteristics of greater reliance on mycorrhizal fungi (Tao and Zhiwei, 2005).

Conclusions

In *J. curcas*, *Glomus* seems to be the most abundant species of AMF and the genotype this plant may influence the AMF community. Regardless of the AMF community present in the area of cultivation, these plants present a high percentage of mycorrhizal colonization and high number of spores in their rhizosphere. The joint use of morphological and molecular methods for identification of AMF species provides more complete information about the diversity of AMF present in the rhizosphere of plants in the field.

Conflict of Interest

The author(s) did not declare any conflict of interest.

ACKNOWLEDGEMENTS

The authors are very grateful to the following Brazilian Financial Institutions: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do estado de Minas Gerais (FAPEMIG). We are also grateful to the Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG), Unidade Regional Norte de Minas (URENM).

REFERENCES

Achten WMJ, Verchor L, Franken YJ, Mathijs E, Singh VP, Aerts R, Muys B (2008). Jatropha Bio-diesel production and use. Biomass Bioenerg. 32: 1063-1084.

Alguacil MM, Torrecilla E, Hernández G, Roldán A (2012). Changes in the diversity of soil arbuscular mycorrhizal fungi after cultivation for biofuel production in a Guantanamo (Cuba) Tropical System. PLoS ONE. 7(4): e34887.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids. Res. 25: 3389-3402.

Augé RM (2001). Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. Mycorrhiza 11: 3-42.

Behera SK, Srivastava P, Tripathi R, Singh JP, Singh N (2010). Evaluation of plant performance of *Jatropha curcas* L. under different agro-practices for optimizing biomass- A case study. Biomass Bioenerg. 34: 30-41.

Berbara RLL, Souza FA, Fonseca HMAC (2006). Fungos micorrízicos arbusculares: muito além da nutrição, in: Fernandes MS (Ed.), Nutrição Mineral de Plantas. Sociedade Brasileira de Ciência do Solo, Viçosa, pp. 53-85.

Charoenpakdee S, Phosri C, Dell B, Lumyong S (2010). The mycorrhizal status of indigenous arbuscular mycorrhizal fungi of physic nut (*Jatropha curcas*) in Thailand. Mycosphere. 1(2): 167-181.

Cornejo P, Azcón-Aguilar C, Barea JM, Ferrol N (2004). Temporal Temperature Gradient Gel Electrophoresis (TTGE) as a tool for the characterization of arbuscular mycorrhizal fungi. FEMS Microbiol. Lett. 241: 265-270.

De Souza FA, Kowalchuk GA, Leeflang P, Van Veen JA, Smith E (2004). PCR-denaturing gradient gel electrophoresis profiling of inter- and intraspecies 18S rRNA gene sequence heterogeneity in an arbuscular mycorrhizal fungi of the genus *Gigaspora*. Appl. Environ. Microbiol. 70: 1413-1424.

Demirbas A (2007). Importance of biodiesel as transportation fuel. Energ. Policy. 35: 4661-4670.

Douds Jr. DD, Millner PD (1999). Biodiversity of arbuscular mycorrhizal fungi in agroecosystems. Agric. Ecosyst. Environ. 74: 77-93.

Douhan GW, Petersen C, Bledsoe CS, Rizzo DM (2005). Contrasting root associated fungi of three common oak-woodland plant species based on molecular identification: host specificity or non-specific amplification? Mycorrhiza 15: 365-372.

Drumond MA, Santos CAF, Oliveira VR, Martins JC, Anjos JB, Evangelista MRV (2009). Desempenho agronômico de genótipos de pinhão-manso no semiárido pernambucano. Cienc. Rural. 40: 44-47.

Gerdeman JW, Nicholson TH (1963). Spores of mycorrhizal endogene extracted from soil by wet sieving and decanting. T. Brit. Mycol. Soc. 46: 235-244.

Giovannetti M, Mosse B (1980). An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol. 84: 489-500.

Hazard C, Gosling Paul, Gast CJ, Mitchell DT, Doohan FM, Bending GD (2013). The role of local environment and geographical distance in determining community composition of arbuscular mycorrhizal fungi at the landscape scale. ISME J. 7: 498-508.

Helgason T, Fitter AH, Young JPW (1998). Ploughing up the wood-wide web? Nature 394-431.

Helgason T, Fitter AH (2005). The ecology and evolution of the arbuscular mycorrhizal fungi. Mycologist 19: 96-101.

Hempel S, Renker C, Buscot F (2007). Differences in the species composition of arbuscular mycorrhizal fungi in spore, root and soil communities in a grassland ecosystem. Environ. Microbiol. 9(8): 1930-1938.

Hernández-Montiel LG, Rueda-Puente EO, Cordoba-Matson MV, Holguín-Peña JR, Zulueta-Rodríguez R (2013). Mutualistic interaction of rhizobacteria with arbuscular mycorrhizal fungi and its antagonistic effect on *Fusarium oxysporum* in Carica papaya seedlings. Crop Prot. 47: 61-66.

Ho I (1987). Vesicular arbuscular mycorrhizae of halophytic grasses in the Alvord desert of Oregon. Northwest Sci. 61: 148-151.

INVAM (2010). International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi. Available at http://invam.caf.wvu.edu/. Acessed 10 September 2010.

Jongschaap REE, Corre WJ, Bindraban PS, Brandenburg WA (2007).

Claims and Facts on *Jatropha curcas* L.: Global *Jatropha curcas* evaluation, breeding and propagation programme. Plant Research International Report, Wageningen, Laren.
Kowalchuk GA, De Souza FA, Van Veen JA (2002). Community analysis of arbuscular mycorrhizal fungi associated with *Ammophila arenaria* in Dutch coastal sand dunes. Mol. Ecol. 11:571-581.
Liang Z, Drijber RA, Lee DJ, Dwiekat IM, Harris SD, Wedin DA (2008). A DGGE-cloning method to characterize arbuscular mycorrhizal community structure in soil. Soil Biol. Biochem. 40: 956-966.
Ma WK, Siciliano SD, Germida JJ (2005). A PCR-DGGE method for detecting arbuscular mycorrhizal fungi in cultivated soils. Soil Biol. Biochem. 37:1589-1597.
Morton JB (1988). Taxonomy of VA mycorrhizal fungi: classification, nomenclature, and identification. Mycotaxon. 32: 267-324.
Muthukumar T, Udaiyan K (2002). Seasonality of vesicular-arbuscular mycorrhizae in sedges in a semi-arid tropical grassland. Acta Oecol. 23:337-347.
Oehl F, Laczko E, Bogenrieder A, Stahr K, Bösch R, Heijden MV, Sieverding E (2010). Soil type and land use intensity determine the composition of arbuscular mycorrhizal fungal communities. Soil Biol. Biochem. 42:724-738.
Openshaw K (2000). A Review of *Jatropha curcas*: an oil plant of unfulfilled promise. Biomass Bioenerg. 19: 1-15.
Öpik M, Moora M, Liira J, Kõljalg U, Zobel M, Sen R (2003). Divergent arbuscular mycorrhizal fungal communities colonize roots of *Pulsatilla* spp. in boreal Scots pine forest and grassland soils. New Phytol. 160: 581-593.
Öpik M, Moora M, Zobel M, Saks Ü, Wheatley R, Wright F, Daniell T (2008). High diversity of arbuscular mycorrhizal fungi in a boreal herb-rich coniferous forest. New Phytol. 179:867-876.
Öpik M, Vanatoa A, Vanatoa E, Moora M, Davison J, Kalwij JM, Reier Ü, Zobel M (2010). The online database MaarjAMreveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). New Phytol. 188:223-241.
Phillips JM, Hayman DS (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. T. Brit. Mycol. Soc. 55:158-161.
Porras-Soriano A, Soriano-Martín ML, Porras-Piedra A, Azcón R (2009). Arbuscular mycorrhizal fungi increased growth, nutrient uptake and tolerance to salinity in olive trees under nursery conditions. J. Plant Physiol. 166: 1350-1359.
Pouyu-Rojas E, Siqueira JO, Santos JGD (2006). Compatibilidade simbiótica de fungos micorrízicos arbusculares com espécies arbóreas tropicais. Rev. Bras. Ciênc. Solo. 30:413-424.
Pozo MJ, Cordier C, Dumas-Gaudot E, Gianinazzi S, Barea JM, Azcón-Aguilar C (2002). Localized versus systemic effect of arbuscular mycorrhizal fungi on defense responses *Phytophthora* infection in tomato plants. J. Exp. Bot. 53(368):525-534.
Pozo M, Azcón-Aguilar C (2007). Unraveling mycorrhiza-induced resistence. Curr. Opin. Plant. Biol. 10:393-398.
Redecker D, Schüßler A, Stockinger H, Stürmer SL, Morton JB, Walker C (2013). An evidence-based consensus for the classification of arbuscular mycorrhizal fungi (Glomeromycota). Mycorrhiza 23: 515-531.
Rodríguez-Echeverría S, Freitas H (2006). Diversity of AMF associated with *Ammophila arenaria* ssp. Arundlnacea in portuguese sand dunes. Mycorrhiza. 16:543-552.
Rozpadek P, Wezowicz K, Stojakowska A, Malarz J, Surówka E, Sobczyk Ł, Anielska T, Wazny R, Miszalski Z, Turnau K (2014). Mycorrhizal fungi modulate phytochemical production and antioxidante activity of *Cichorium intybus* L. (Asteraceae) under metal toxicity. Chemosphere. 112:217-224.
Sanders IR, Croll D (2010). Arbuscular Mycorrhiza: The challenge to understand the genetics of the fungal partner. Annu. Rev. Genet. 44: 271-292.
Schenck NC, Perez Y (1990). Manual for the Identification of VA Mycorrhizal Fungi, third ed. Synergistic Publications, Gainesville, FL, USA.
Schmook B, Serralta-Peraza L (1997). *Jatropha curcas*: Distribution and uses in the Yucatan peninsula of México, in: Gübitz GM, Mittembach M, Trabi M (Eds) Biofuels and Industrial products from *Jatropha curcas*. Dbv-Verlag für die Tecnisch Universität Graz, Graz, Austria, pp. 53-57.
Sharma YC, Singh B (2009). Development of biodiesel: Current Scenario. Renew Sust. Energ. Rev. 13: 1646-1651.
Silva MCS, Mendes IR, Paula TA, Luz JMR, Cruz C, Bazzolli DMS, Kasuya, MCM (2014). Dynamics of arbuscular mycorrhizal fungi in *eucalyptus globulus* plantations. Eur. J. Agric. For. Res. 2: 25-42.
Simon L, Lalonde M, Bruns TD (1992). Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. App. Environ. Microbiol. 58: 291-295.
Smith SE, Read DJ (1997). Mycorrhizal symbiosis. Academic Press, Cambridge, MA. 605 p.
Smith SE, Facelli E, Pope S, Smith FA (2010). Plant performance in stressful environments: interpreting new and established knowledge of the roles of arbuscular mycorrhizas. Plant Soil 326: 3-20.
Tao L, Zhiwei Z (2005). Arbuscular mycorrhizas in a hot and arid ecosystem in southwest China. Appl. Soil Ecol. 29:135-141.

academicJournals

Vol. 9(15), pp. 1075-1081, 15 April, 2015
DOI: 10.5897/AJMR2014.7254
Article Number: 2A7A51652641
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

The role of phenolic compounds in the defense of sooty mold of olive leaves (*Olea europea* L.)

Faiza Ilias[1,3*], Sarra Bensehaila[2], Kenza Medjdoub[3], Imad El Haci[4] and Nassira Gaouar-Benyelles[3]

[1]Center University of Belhadj Bouchaib, Ain Temouchent, Algeria.
[2]Laboratory of Naturelles Bio-Ressources, University of Hassiba Benbouali, Chelef, Algeria.
[3]Laboratory of Ecology and Management of Ecosystems, Department of Biology, University of Tlemcen, Tlemcen 13000, Algeria.
[4]Laboratory of Natural Products, Department of Biology, University of Tlemcen, Tlemcen 13000, Algeria.

Received 6 November, 2014; Accepted 13 March, 2015

The aim of this study is to demonstrate the role of phenolic compounds in the olive leaves infected by sooty mold. The results show that the presence of sooty mold induces a high production of polyphenols in infected leaves of olive compared to the uninfected ones. The high concentrations of flavonoids and alkaloids in the infected trees suggest that they make the olive tree resistant to this fungi disease. Analyses by high-performance liquid chromatography (HPLC) confirmed the presence of verbascoside acid, oleuropeinacid, caffeic acid and for the first time, tannic acid. These substances are good resistance markers and should help to make efficient strategies for the bio-control of this kind of fungal disease.

Key words: *Olea europea* L., fungi, phenolic compounds, defense, high-performance liquid chromatography (HPLC).

INTRODUCTION

The olive tree, *Olea europea* is in full expansion in many countries. Despite its importance, it faces several diseases that severely affect its tree production (Santos et al., 2013), one of which is sooty mold. It is accepted that sooty mold is a complex of dark-pigmented fungi of several genera, which have been described as non parasitic, saprophytic, and superficial on plants (Reynolds, 1999; Jouraeva et al., 2006). This fungal complex covers both leaf surfaces and small branches, giving a black aspect to the olive tree (Reynolds, 1999).

The black scale insect, *Saissetia oleae* (Olivier) (Hemiptera: Coccidae) excretes honeydew, facilitates the installation and growth of multiple fungi that cover the olive leaves and supports the proliferation of the sooty mold (Passos-Carvalho et al., 2003; Jouraeva et al., 2006). Heavy infestation reduces photosynthetic activity (Haniotakis, 2005) and a consequent alteration of the normal metabolism and physiology of the plant and ultimately its growth (Santos et al., 2013). For example, Passos-Carvalho et al. (2003) mentioned the negative effects of sooty mold on parameters such as photo-synthesis, chlorophyll, and respiration of the olive tree.

Corresponding author. E-mail: faizahope@yahoo.fr.

Plants have complex mechanisms to protect themselves against pathogens. Phenolic secon-dary metabolites, which are involved in the special organoleptic properties of oil, have been shown to play a role in the resistance of some olive (*O. europea L.*) varieties to oil autoxidation (Botia et al., 2001). In addition, some reports (Marsilio and Lanza, 1998) have shown that some phenolic substances of olive trees may inhibit the growth of bacteria, such as *Lactobacillus plantarum*, *Leuconosto cmesenteroides* and fungi like *Phytophthora* (Del Rio et al., 2003). Similarly, the phenolic metabolism of the olive tree is considered as a plant-response to the infection caused by *Verticillium dahliae* (Daayf, 1993). Thus, increasing the endogenous levels of these secondary metabolites can improve the resistance properties of the plant and can be used as a natural alternative for preventing plant diseases. Methods for detecting and recognizing phenolic compounds rely mainly on chromatographic separation, using HPLC analyses (El Modafar and El Boustani, 2001) which allow their successful identification.

In Algeria, little is known about the resistance of *O. europea* L. The aim of this work is to isolate the pathogens involved in the sooty mold and detect the phenolic compounds potentially present in the defense of *O. europea* L. with the chemical nature of these compounds using HPLC method.

MATERIALS AND METHODS

Ten randomly selected olive trees of the Sigoise cultivar were sampled in an olive grove located in Tlemcen (in the northeast of Algeria). In each tree, 5 current season branch segments with visible sooty mold coverage and 5 healthy branch segments were detached from the south-facing canopy at about the same elevation (2.0 m). Branches were taken to a growth chamber at 22°C, placed in a container with water, and left overnight to avoid dehydration.

Isolation of the pathogen

To isolate the pathogens involved in the sooty mold disease, leaf samples were treated according to the method of Pinto (2007); they were placed on potato dextrose agar (PDA) medium amended with streptomycin (100 ppm) and incubated at 25°C in the dark, for 7 days.

Yields extraction

The leaves were washed and dried with paper towel; they were cut into approximately 1 cm squares, dried in an oven at 60°C for at least 24 h, crushed and degreased in a soxhlet, before use. All analyses were conducted in triplicate, and the results were based on dry weight per 100 g of sample.

Tannins extraction

Powdered material (100 g) was extracted at 4°C using 500 ml of a mixture of acetone-water (25/45, v/v) for 4 days (Bruneton, 1999). The extracts were filtered under vacuum through filter paper and the acetone was evaporated under reduced pressure. Subsequently, dichloromethane (2 × 25 ml) was used for the extraction of lipids and pigments from the aqueous extracts using a separating funnel. Afterward, the aqueous phase was extracted with 25 ml of ethyl acetate. This process was repeated four times. After filtration, the organic phases (ethyl acetate) containing tannins were recovered and concentrated to dryness under vacuum, using a rotary evaporator. The residue obtained after evaporation was kept at 4°C and used for further investigation.

Flavonoids extraction

A quantity of 10 g of dried material was extracted with 100 ml of methanol and 5 g of calcium carbonate by boiling for 1 h (Danguet and Foucher, 1982). After filtration, through Whatman filter paper, the methanol was evaporated under reduced pressure to eventually give an aqueous extract. Subsequently, the dry extract was recovered with 50 ml of boiling water. The aqueous extract was filtered and subjected to solvent fractionation; firstly with diethyl ether, then ethyl acetate and finally n-butanol, using separating funnel (pyrex). All fractions were concentrated, dried to constant weight in an oven at 45°C and kept at 4°C.

Extraction of alkaloids

An amount of 10 g of dried sample was mixed with 250 ml of HCl 2% and 110 ml of ethyl acetate. After cold soaking (4 °C) for 10 h, the mixture was filtered and basified with NH_4OH. The basic aqueous phase was extracted twice with ethyl acetate until no alkaloid was detected in the aqueous phase. The alkaloid residue was obtained by decantation and evaporation of the organic phase (Bruneton, 1999).

Plant extraction

The dried powder of olive leaves (10 g) was extracted in triplicate, using EtOH (96% v/v) at room temperature, under stirring. The aqueous suspension of the concentrated EtOH extract was evaporated to dryness and used for all investigations (Kukic et al., 2008).

Determination of total phenolic content

The amount of total phenolic content was determined by Folin-Ciocalteu procedure (Singleton and Rossi, 1965). Aliquot (0.1 ml) of each sample extract was transferred into the test tubes and their volumes were made up to 3 ml with distilled water. After addition of 0.5 ml Folin-Ciocalteu reagent and 2 ml of 20% aqueous sodium carbonate, tubes were vortexed and incubated at room temperature under dark condition. The absorbance was recorded after 1h at 650 nm JEN WAY 6405 UV/Vis spectrophotometer. The total phenolic content was calculated as a Pyrocatechol equivalent (mg PE/g DW).

High performance liquid chromatography (HPLC)

Total phenolics analyses on methanolic extract of infected olive leaves were carried out using Jasco HPLC. It consists (Jasco HPLC) of a pump (PU-2089 Plus) and UV detector model UV-2077 with ChromNAV on a XBridge analytical column (RP-C18 : 5 µm, 4.6 x 150 mm) (Waters Inc. USA), having gradient solvent system

Table 1. Gradient solvent composition in HPLC used in total phenolics analyses.

Time (min)	Composition (%)	
	Solvent A (ACN)	Solvent B (H_2O, pH 2.5)
Initial	2.0	98.0
5.00	2.0	98.0
15.00	5.0	95.0
17.00	100.0	0.0
35.00	100.0	0.0
Flow rate (ml/min)	0.7	
Method time	35 min	

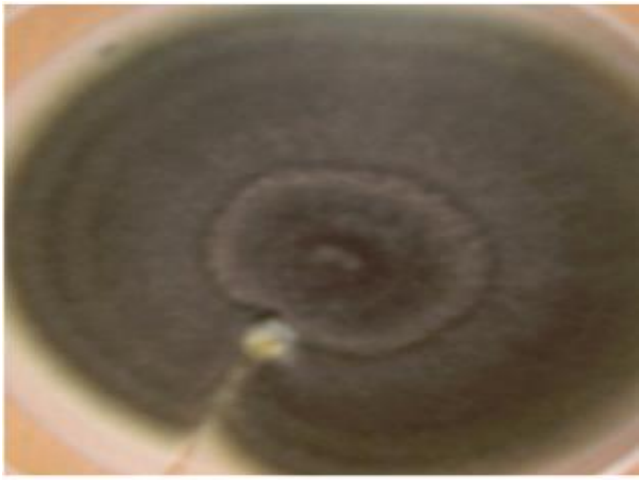

Figure 1. Colony type of *Alternaria* spp.

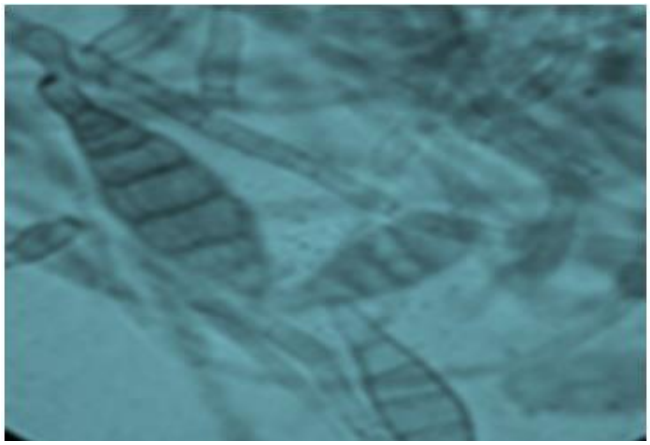

Figure 2. Mycelium and conidiophores of *Alternaria* spp.

and parameter condition as shown in Table 1. The chromatograms were observed at wavelengths of 254, 270, 280 and 329 nm. All the analyses were carried out at sample concentration of 1 mg/ml and injection volume of 20 µl.

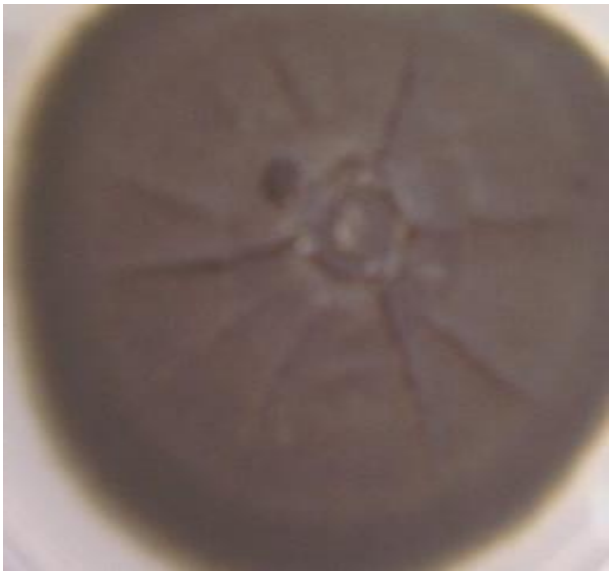

Figure 3. Colony type of *Ulocladium* spp.

RESULTS

Identification of fungi

Leaves with sooty mold showed a dark color covering large areas of both surfaces. The symptoms from infected leaves were very similar to those described for sooty mold of olive tree. Fungal colonies present in our leaves as sooty mold are: *Alternaria* spp. (Figure 1 and 2), *Ulocladium* spp. (Figures 3 and 4) and *Penicillium* spp. (Figures 5 and 6). Mycelia settle on the surface of leaves to form a black film which causes premature aging by suffocation, blocking of photosynthesis and decreasing of gas exchange. It slows growth and leaves a black layer on leaves; it makes xylem to become brown and leaves roll to their inner face, with color changing from yellow to brown. These funguses are responsible for sooty mold in Algeria with the high dispersion of their spores.

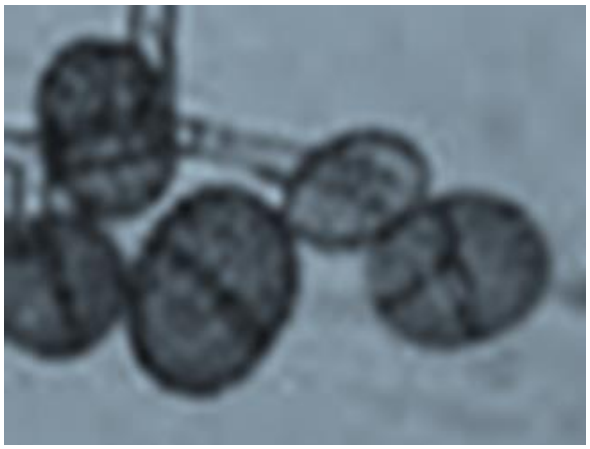

Figure 4. Mycelium and condiophores of *Ulocladium* spp.

Figure 5. Colony type of *Pinicillium* spp.

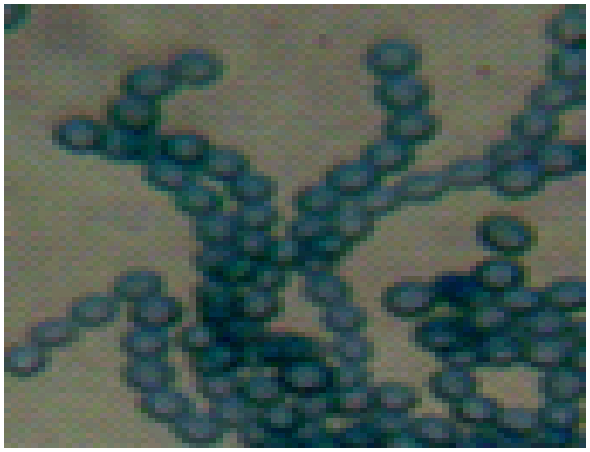

Figure 6. Mycelium and conidiophores of *Penicillium* spp.

Total phenol content

Figure 7 shows the total phenol content in a whole leaf from uninfected and infected olive plants. The total phenol contents in the infected plants (40.8 mg/g) were practically higher than those measured in the uninfected plants (21.7 mg/g).

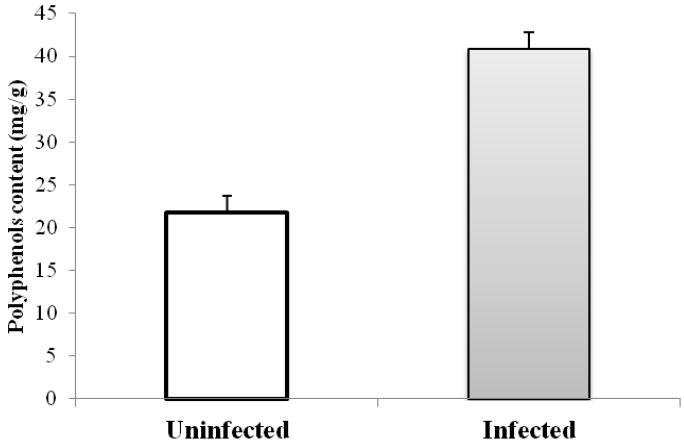

Figure 7. Polyphenols content of uninfected and infected leave of olive.

Yields extraction

The yields of tannins, flavonoids and alkaloids are presented in Figure 8. The yield of tannins in whole leaf from infected and uninfected olive plants was 1.18 and 2.3%, respectively. The yield of flavonoids and alkaloids was higher in infected plants: 4.05 and 2.1% for flavonoids and 2.56 and 1.1% for alkaloids content in uninfected and infected plants, respectively.

Identification of phenolic compounds by HPLC

The data (retention time, λ max in the visible region, and tentative identification) obtained for the phenolic compound peak in the HPLC- analyses are presented in Table 2 and Figure 9. HPLC studies point to four phenolic compounds determined in olive leaves extracts: caffeic (*tR* = 9.850 min, maximum absorbance at 249 nm), Verbascoside (*tR* = 11.419 min, maximum absorbance at 240 nm), tannic (*tR*= 18.41 min, maximum absorbance at 253 nm) and oleuropein (*tR* = 20.06 min, maximum absorbance at 233 nm).

DISCUSSION

The highest numbers of fungal species causing fruit rot of olive are common saprophytes or secondary invaders that normally penetrate through injuries made by biotic or abiotic factors (Lazzizera et al., 2008). However, this is the first report of *Alternaria* spp*., Ulocladium* spp*.* and

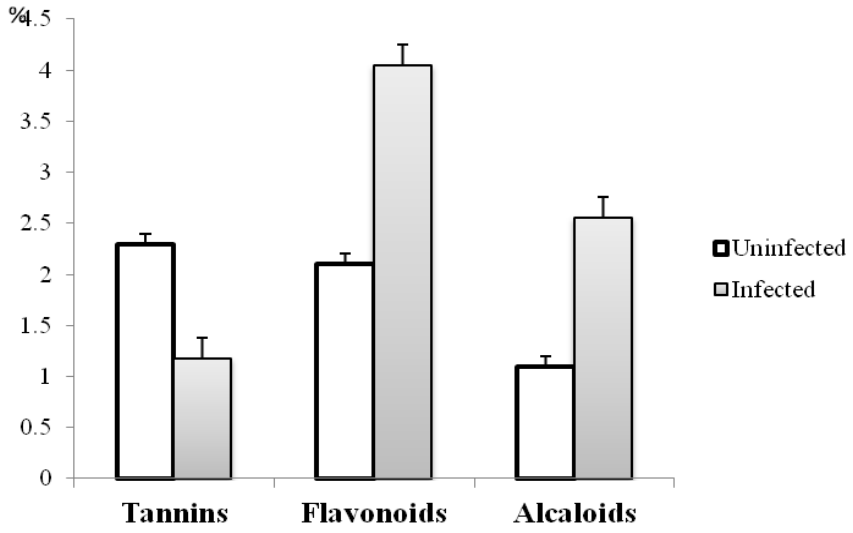

Figure 8. Tannins, flavonoids and alkaloids contents of uninfected and infected leave of olive.

Table 2. Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax) and tentative identification of phenolic compounds in olive leaves.

Peak	Rt (min)	Λmax (nm)	Tentative identification
1	0.650	243	N.D
2	2.022	250	N.D
3	2.538	252	N.D
4	9.850	249	Caffeic
5	11.419	240	Verbascoside
6	18.41	253	Tannin
7	20.06	233	Oleuropein

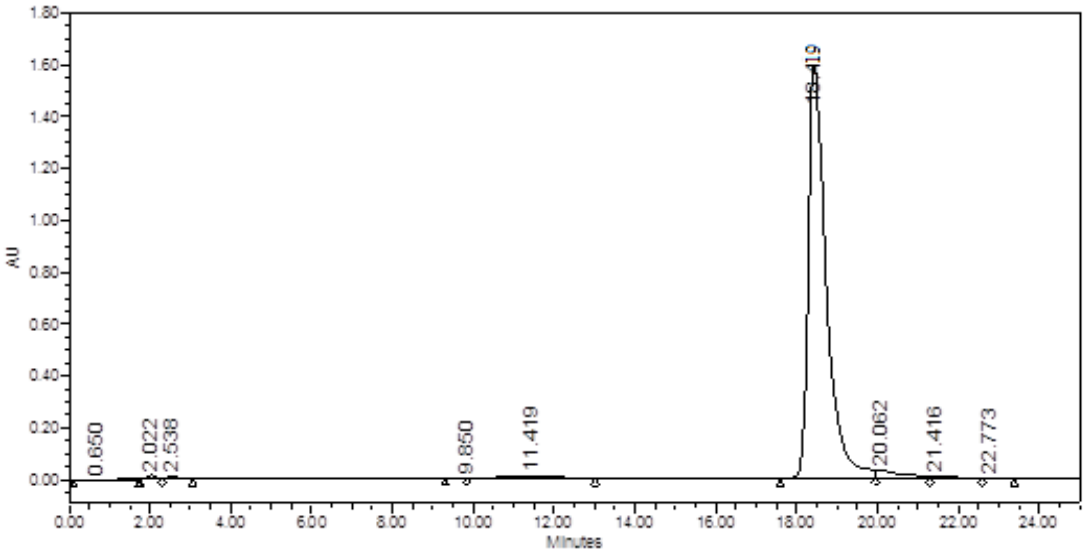

Figure 9. Chromatogram (zoom) recorded at 254 nm showing the phenolic compounds profiles identified and not identified of olive leaves (*Olea europea* var. Sigoise).

Penicillium spp. causing leaf chlorosis and olive fruit rot in Algeria.

The obtained results show that total polyphenols were present in infected olive trees at higher levels than in uninfected olive trees; and the difference was statistically significant ($p< 0.001$). However, the difference in tannin and flavonoid yields was significant ($p < 0.05$).

This is certainly due to the type of analysis which shows that the total polyphenols synthesis was better after infection by the fungi. The total polyphenols content obtained confirms this idea because the total polyphenols analysis gives a quantitative result, whereas the yield gives a qualitative one.

The inoculation of the olive twigs by a conidial suspension of *Verticillium dahlia* resulted in important modifications in flavone and phenol levels (El Boustani et al., 1998). These findings suggest that the first step of the response mechanism to infection in olive plants is a rapid accumulation of phenols at the infection site, thus reducing or slowing the pathogen growth, as reported for other vegetal materials (Del Rio et al., 2004). Therefore, in contrast to flavonoids and alkaloids, the tannin content of the uninfected sample was higher than that of the infected one. Our results are in agreement with those of Corbaz (1990), whose study results show that the young leaves at the cotton plant are often resistant to *V. dahlia* and become sensitive as they grow older. This phenomenon might be ascribed to the inhibition of mycelium growth in young tissues, which contain higher concentrations of substances such as tannins than those in the old leaves.

In selective extractions, those concentrations of alkaloids in infected olive plants were higher than in uninfected ones also suggest that alkaloids may have a role in the response mechanism of olive plants to sooty mold. These results are similar to that found by Bensalah et al. (2014) who found olive infected by *V. dahliae*.

Our main findings were that the HPLC analyses revealed the presence of some phenolic compounds in infected olive leaves, namely verbascoside and tannic acid. Bensalah et al. (2014) found verbascoside for the first time in olive leaves infected by *V. dahliae*. This result confirms that verbascoside compounds have a role in the resistance or defense of olive against fungi attacks. Tannic acid is reported to have a role in the resistance of plant to insects. In this study, we have found that this acid has a role in the defense mechanism to sooty mold. We suggest that this compound have a role or function in the resistance to fungi.

Conclusion

This study strongly suggests that some of phenolic compounds present in olive leaves variety Sigoise play a role in the natural defense mechanism, as it has been established for other phenolic secondary metabolites in different plant materials infected by pathogenic fungi. The HPLC analysis revealed the presence of new phenolic compounds, namely tannin.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Bensalah F, Gaouar-Benyelles N, Choukri Beghdad M (2014). High-performance liquid chromatography (HPLC) Identification of five new phenolic compounds involved in the olive tree (*Olea europea var*. Sigoise) resistance to *Verticillium dahliae*. Afr. J. Microbiol. 8 (2): 192-199. DOI: 10.5897/AJMR2013.5844.

Botia JM, Ortuno A, Benavente-Garcia O, Baidez AG, Frias J, Marcos D, Del Rio JA (2001). Modulation of the biosynthesis of some phenolic compounds in *Oleaeuropea*L. fruits: their influence on olive oil quality. J. Agric. Food Chem. 49: 355-358.

Bruneton J (1999). Pharmacognosie. Phytochimie. Plantes médicinales. Technique & Documentation. 3ème Ed. Lavoisier, Paris, pp. 370-401.

Corbaz R (1990). Principes de phytopathologie et de lutte contre les maladies des plantes. Ed. polytechniques and university romandes. pp. 103-226.

Daayf F (1993). La verticilliose du cotonnier. Pouvoir pathogène et diversité génétique de *Verticillium dahliae*, réactions de la plante à l'infection. Phd, University of Montpellier, France. pp. 14-20.

Danguet JC, Foucher JP (1982). The flavonoides of *Arbutusunedo*L. Medicals and phytotherapiticals Plants. 16(3):185-191.

Del Rio JA, Baidez AG, Botia JM, Ortuno A (2003). Enhancement of phenolic compounds in olive plants (*Oleaeuropea*L.) and their influence on resistance against *Phytophtora*sp. Food. Chem. 83: 75-78.

Del Rio JA, Gomez P, Baidez AG, Arcas MC, Botia JM, Ortuno A (2004). Changes in the levels of polymetoxy flavones and flavonones as a part of the defence mechanism of *Citrus sinensis* (cv. Valencia late) fruits against *Phytophtora citrophtora*. J. Agric. Food. Chem. 52: 1913-1917.

El Boustani, El Modafar C, Boulouha B, Serrhini M(1998). 2nd International Electric Conference of Synthetic Organic Chemistry, personal communication).

El Modafar C, El Boustani E (2001). Cell wall-bound phenolic acid and lignin contents in date palm as related to its resistance to Fusarium oxysporum. Biol. Plant, 44:125-130.

Haniotakis G (2005). Olive pest control: Present status and prospects. IOBC/wprs Bulletin. 28:1-9.

Jouraeva VA, Johnson DL, Hassett JP, Nowak DJ, Shipunova NA, Barbarossa D (2006). Role of sooty mold fungi in accumulation of fine-particle-associated PAHs and metals on deciduous leaves. Environ. Res. 102:272-282.

Kukic J, Popovic V, Petrovic S, Mucaji P, Ciric A, Stojkovic D, Sokovic M (2008). Antioxidant and antimicrobial activity of Cynaracardunculus extracts. Food. Chem. 107:861-868.

Lazzizera C, Frisullo S, Alves A, Phillips AJL (2008). Morphology, phylogeny and pathogenicity of *Botryos phaeria* and *Neofusicoccum* species associated with drupe rot of olives in southern Italy. Plant. Pathol. 57:948-956.

Marsilio V, Lanza B (1998). Characterisation of an oleuropein degrading strain of *Lactobacillus plantarum*. Combined effects of compounds present in olive fermenting brines (phenols, glucose and NaCl) on bacterial activity. J. Sci. Food. Agric. 76:520-524.

Passos-Carvalho J, Torres LM, Pereira JA, Bento AA (2003). A cochonilha-negra *Saissetia oleae* (Olivier, 1791) (Homoptera: Coccidae). Lisbon, Portugal: INIA/UTAD/ESAB (in Portuguese).

Pinto G (2007). Regeneracao de plantas de *Eucalyptus globulus* por embriogenese somatica. PhD, University of Aveiro, Aveiro, Portugal (in

Portuguese).
Reynolds DR (1999). *Capnodium citri*: the sooty mold fungi comprising the taxon concept. Mycopathologia 148: 141-147.
Santos SAP, Santos C, Silva S, Pinto G, Laura M. Torres LM, Nogueira AJA (2013). The effect of sooty mold on fluorescence and gas exchange properties of olive tree. Turk. J. Biol. 37:620-628.
Singleton VL, Rossi JA (1965). Colorimetry of total phenolics with Phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Viticulture, 16:144-148.

academicJournals

Vol. 9(15), pp. 1082-1088, 15 April, 2015
DOI: 10.5897/AJMR2013.5573
Article Number: 8909C6752643
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Reduced intracellular drug accumulation augments fluoroquinolone and β-lactam drugs resistance in clinical Gram negative bacteria from Nigeria

D. Olusoga Ogbolu[1,2]*, O. A. Daini[3], A. O. Terry Alli[1] and M. A. Webber[2]

[1]Department of Biomedical Sciences, Ladoke Akintola University of Technology, Ogbomoso, Osogbo Campus, Osun State, Nigeria.
[2]Antimicrobial Research Group, Immunity and Infection, Institute of Biomedical Research, University of Birmingham, Birmingham, UK.
[3]Biochemistry Department, Olabisi Onabanjo University, Remo Campus, Ago-Iwoye, Ogun State, Nigeria.

Received 11 February, 2013; Accepted 18 July, 2014

In Nigeria, quinolones and β-lactam antibiotics are widely used as broad-spectrum antibiotics to treat infections caused by various Gram-negative pathogens. The outer membrane is the major permeability barrier limiting target access to quinolones and other drugs in Gram-negative bacteria. This study aimed to identify the role of outer membrane porins (OMPs) and uptake in fluoroquinolone (FQ) and β-lactam drugs accumulation. In total, 134 non-duplicate, Gram-negative bacilli isolates of 13 species from different hospitals were investigated for susceptibility to a panel of antibiotics, including loss of outer membrane porins and measuring active efflux. The minimum inhibitory concentrations (MIC) results showed level of resistance to many antibiotics was extremely high having MIC_{90} value of 256 µg/ml or higher for all drugs, most importantly fluorquinolones, ciprofloxacin; sparfloxacin or third generation cephalosporin, ceftazidime; ceftriaxone. SDS-PAGE revealed different outer membrane porin (OMP) profiles on the basis of relative mobility among the strains. The majority of the isolates lack OMPs. The steady-state concentration of drug taken up by the isolates was measured; most of the strains accumulate less bis-benzimidine than the control strain, *Salmonella enterica* L354. The isolates from University College Hospital, Ibadan accumulate fewer drugs and they are more resistant with high minimum inhibitory concentrations when compared with the rests of the hospitals. Active efflux either singly or in tandem with OMPs alterations could be responsible for the low accumulation of fluoroquinolone and β-lactam antibiotics seen in this study and their increased resistance to both important classes of antibiotics.

Key words: Fluoroquinolone, β-lactam, accumulation, resistance and Gram negative, bacteria.

INTRODUCTION

Chromosomal resistance to fluoroquinolones (FQs) due to amino acid substitutions in the quinolone resistance-determining regions (QRDRs) of DNA gyrase (GyrA) and/or topoisomerase IV (ParC) has been reported

*Corresponding author. E-mail: olusogadave@yahoo.com. Tel: +2347055776547.

(Jacoby, 2005), and has also been found in Gram negative bacteria from Nigeria (Ogbolu et al., 2011). Plasmid-mediated mechanisms of resistance to FQs mediated by qnr alleles (qnrB, qnrS, qnrC and qnrD), the aac(6')-Ib-cr variant and qepA have been identified from many countries (Robicsek et al., 2006). Decreased activity of FQ against *Escherichia coli* has also been related to a reduced intracellular drug accumulation due to lipopoly-saccharide or porin alterations impairing uptake or because of enhanced efflux (Hirai et al., 1986; Hooper et al., 1986). The outer membrane is the major permeability barrier limiting target access to quinolones and other drugs in Gram-negative bacteria. Quinolones can penetrate the outer membrane of *E. coli* not only by diffusion through the OmpF and OmpC porin channels (Hirai et al., 1986) but also by diffusion through the phospholipids layer (Bedard et al., 1987; Chapman and Georgopapadakou, 1988).

Therefore, several mutations that modify outer membrane structural components can lead to quinolone resistance. These types of mutations are associated with low-level resistance to quinolones and cross resistance to other groups of antibiotics that use the same pathways across the outer membrane (Wolfson and Hooper, 1985). Also, association of multiple antibiotic resistance genes on mobile genetic elements has been an important mechanism of dissemination of multidrug resistance and may explain in addition the frequent association between FQ resistance and resistance to extended-spectrum β-lactams in *Enterobacteriaceae*. In addition, the presence of multiple resistance genes on a plasmid expands the subset of drugs that may select for dissemination of multidrug resistance plasmids.

In Nigeria, quinolones and β-lactam antibiotics are widely used as broad-spectrum antibiotics to treat infections caused by various Gram-negative pathogens. Ogbolu et al. (2011) reported an extremely high level of resistance to multiple antibiotics, including FQs and cephalosporins, detected amongst a diverse panel of Gram-negative isolates from various hospitals in Nigeria. This resistance was underpinned by the carriage of a wide variety of plasmid-borne quinolone resistance alleles and ESBL genes, including the first identification of the qnrD allele outside of China and CTX-M group of enzymes. In this study, we identified a reduced FQ and β-lactam drugs accumulation in clinical Gram negative bacteria caused by loss of outer membrane porins and decreased uptake.

MATERIALS AND METHODS

Bacterial isolates and their properties

One hundred and thirty-four clinical Gram-negative bacterial isolates of 13 species were obtained from 585 non-duplicate clinical specimens, including aspirates, ear swab, wound swab, throat swab, high vaginal swab, eye swab, sputum, urine, catheter tip, cerebrospinal fluid and blood culture, for the period, 2005-2007. Single isolates from each specimen were retained. Isolates were from four teaching hospitals in South-Western Nigeria, namely University College Hospital (Ibadan), Obafemi Awolowo University Teaching Hospital (Ile-Ife), Ladoke Akintola University of Technology Teaching Hospital (Osogbo) and Olabisi Onabanjo University Teaching Hospital (Sagamu). All isolates were identified using API 20E strips (bioMérieux, Marcy l'Etoile, France).

Our previous study showed that mutations were found within gyrA and additional mutations within parC for quinolones resistance isolates. This included carriage of a variety of transferable quinolone resistance alleles *qnrA1, qnrB, qnrD*, *aac(6')-Ib-cr* and *qepA*. For β-lactam resistance, *CTX-M-15* was found predominantly, only 1 was *CTX-M-3*. The presence of *ampC* genes was indicated for a number of strains phenotypically and was confirmed in six isolates by PCR. Sequencing identified these genes as *ACT-1, DHA-1* and *CMY-2*. These genes were found in isolates co-producing other extended-spectrum β-lactamase (ESBL) genes such as *CTX-M, TEM, SHV* and *OXA* genes (Table 1) (Ogbolu et al., 2011).

Determination of antibiotic susceptibility

Antimicrobial disc susceptibility tests were carried out on the isolates using freshly prepared Mueller-Hinton agar (Oxoid, England), 0.5 Macfarland of inoculum was used and standardised by the method of Clinical and Laboratory Standard Institute (CLSI, 2012). The following antibiotic discs were used; pefloxacin, 30 µg; sparfloxacin, 30 µg; ciprofloxacin, 5 µg; ceftriaxone, 30 µg; ceftazidime, 30 µg; amoxicillin, 25 µg; amoxicillin/clavulanic acid, 20/10 µg; gentamicin, 10 µg; tetracycline, 30 µg; nalidixic acid, 30 µg. All susceptibility testing runs included the control organisms *E. coli* NCTC 10418 and *Pseudomonas aeruginosa* NCTC 10662. Plates with antibiotic discs were incubated for 24 h at 37°C and sensitivity pattern was compared with that of the control culture.

Minimum inhibitory concentrations (MICs) of a panel of antibiotics were determined and interpreted using the agar dilution method according to the guidelines of the British Society for Antimicrobial Chemotherapy (BSAC) (http://www.bsac.org.uk/susceptibility testing/guide to antimicrobial susceptibility testing.cfm). The antibiotics tested were gentamicin, tetracycline, amoxicillin, amoxicillin/clavulanic acid (AMC), nalidixic acid, ciprofloxacin, pefloxacin, ofloxacin, sparfloxacin, ceftazidime and ceftriaxone. MIC breakpoints for defining ciprofloxacin susceptibility used were: susceptible, 1 µg/ml; intermediate susceptible, 2 µg/ml; and resistant, 4 µg/ml. All susceptibility testing runs included the control organisms as stated above.

Analysis of outer membrane proteins

Bacterial cells were grown in Mueller-Hinton broth to the logarithmic phase, and were lysed by sonication. Outer membrane proteins (OMPs) were extracted with sodium lauryl sarcosinate (Sigma, UK) and recovered by ultracentrifugation, as described previously (Filip et al., 1973). Protein concentrations were determined with the Bradford protein assay kit (Sigma, UK) as described by the manufacturer. The OMP profiles were determined by SDS-PAGE using 12% SDS gels followed by Coomassie blue staining (Carlsbad, CA). The presence and intensity of bands were visualised when compared with wild type isolates of *E. coli*, I364; *K. pneumoniae*, H43; *P. aeruginosa*, GI using Syngene Image analyser software.

Measuring the activity of active efflux using Hoescht 33342 (bis-benzimide)

The efflux activity of bacteria; test isolates or control, L354 (a wild

Table 1. Properties of bacterial isolates used in the study (Ogbolu et al., 2011).

Species	N	PCR-positive for PMQR genes [n(%)]						PCR-positive for β-lactamase genes [n(%)]			
		qnrA	*qnrB*	*qnrS*	*QnrD*	*qepA*	*aac(6')-Ib-cr*	*TEM*	*SHV*	*OXA*	*CTX-M*
Klebsiella pneumoniae	63	0(0)	0(0)	0(0)	0(0)	1(1.6)	5(21.7)	47(74.6)	9(14.3)	1(1.6)	4(6.3)
Escherichia coli	28	3(10.7)	1(3.6)	0(0)	0(0)	2(7.1)	3(10.7)	24(85.7)	10(35.7)	5(17.9)	9(32.1)
Pseudomonas aeruginosa	13	0(0)	0(0)	0(0)	1(7.7)	0(0)	2(15.4)	12(92.3)	4(30.8)	2(15.4)	3(23.1)
Proteus mirabilis	11	1(9.1)	0(0)	0(0)	2(18.2)	0(0)	7(63.6)	10(90.9)	3(27.3)	3(27.3)	3(27.3)
Pseudomonas oryzihabitans	6	1(16.7)	0(0)	0(0)	0(0)	1(16.7)	3(50)	6(100)	3(50)	0(0)	1(16.7)
Burkholderia cepacia	2	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	2(100)	1(50)	0(0)	1(50)
Aeromonas hydrophilia	1	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)
Enterobacter cloacae	2	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Morganella morganii	3	0(0)	0(0)	0(0)	0(0)	1(33.3)	1(33.3)	2(66.7)	1(33.3)	1(33.3)	1(33.3)
Pseudomonas luteola	1	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)	1(100)	0(0)	0(0)	0(0)
Serratia adorifera	1	0(0)	1(100)	0(0)	0(0)	0(0)	1(100)	1(100)	1(100)	1(100)	1(100)
Stenotrophomonas maltophilia	2	0(0)	1(50)	0(0)	0(0)	0(0)	0(0)	2(100)	1(50)	1(50)	1(50)
Citrobacter freundii	1	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	1(100)	1(100)	1(100)	1(100)

PMQR, plasmid-mediated quinolone resistance.

type *Salmonella enterica* subsp *enterica* serovar Typhimurium) was determined by measuring the accumulation of the fluorescent dye Hoechst 33342 (bis-benzimide; 2.5 µM) + known EPIs (40 mg/L PAßN). Measurements were taken at excitation and emission wavelengths of 350 and 460 nm, respectively, over 30 min using a FLUOstar OPTIMA (BMG Labtech, Aylesbury, UK), as previously described (Webber et al., 2008).

The data were recorded and analysed by averaging each set of triplicate repeats and subtracting the appropriate average blank values e.g. for test isolate 'A' average the values from each well for each time point and subtract the average value from the three PBS with bis- benzimide blank wells for each time point to give a corrected value. Standard deviation for each sample at each data point was also calculated. The data was presented by plotting graphs to include standard deviation.

RESULTS

Susceptibility testing

The *in vitro* susceptibility pattern of all isolates to 10 antibiotics was determined by disc diffusion and data is presented in Table 2. All the strains examined showed resistance to one or more of the ten antibiotics used for the study. The results depict a pattern of multiple and high level resistance; more than 60% of isolates were resistant for each antibiotic. Overall, fluoroquinolones showed slightly lower level of resistance than the rest of the antibiotics including the third generation cephalosporins except nalidixic acid and tetracycline with only 0 - 4% sensitivity to *E. coli, K. pneumoniae* and *P. aeruginosa*. Table 2 also shows the minimum inhibitory concentrations of 10 antibiotics using the agar dilution method. Determination of precise MIC values confirmed the numbers of strains resistant to clinical breakpoint concentrations for all antibiotics. The MIC results also showed that the level of resistance to many antibiotics was extremely high having MIC_{90} value of 256 µg/ml or higher for all drugs.

Loss of outer membrane porins in quinolone resistance

Outer membranes were prepared from cells grown overnight in a medium to induce expression of the outer membrane porins OmpF and OmpC. SDS-PAGE revealed several different OMP profiles among the strains (Figure 1). OMPs were identified on the basis of relative mobility; the majority of the isolates lack both OmpF and OmpC in *E. coli*. The highest MICs of fluoroquinolones and β-lactam were usually, although not always associated with those isolates lacking Omps.

Reduced accumulation of fluoroquinolone was responsible for resistance

The steady-state concentration of drug taken up by the isolates was measured; representatives of the results of experiments are presented in Figure 2. Overall, most of the strains accumulate less bis-benzimidine than L354 wild-type control strain (Figure 2A). Majority of the isolates are multiply drug resistant (MDR) with quinolone

Table 2. Antimicrobial susceptibility of some of the isolates.

Organisms (no of strains)	Antimicrobial agents (µg)	Disc sensitive (%)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	Range (µg/ml)
Escherichia coli N = 28	Ciprofloxacin (5)	32	256	256	0.015-256
	Pefloxacin (5)	25	256	256	0.25-256
	Sparfloxacin (5)	29	256	256	0.25-256
	Ceftazidime (30)	24	32	256	0.25-256
	Ceftriazone (30)	25	8	256	0.25-256
	Augmentin (30)	25	256	256	0.25-256
	Amoxycillin (25)	21	256	256	0.25-256
	Gentamicin (10)	36	256	256	0.25-256
	Tetracycline (30)	4	256	256	0.25-256
	Nalidixic acid (30)	4	>256	>256	1-256
K. pneumoniae ssp *pneumoniae* N = 63	Ciprofloxacin (5)	17	256	256	0.015-256
	Pefloxacin (5)	14	256	256	0.25-256
	Sparfloxacin (5)	29	256	256	0.25-256
	Ceftazidime (30)	10	256	256	0.25-256
	Ceftriazone (30)	8	256	256	0.25-256
	Augmentin (30)	9	256	256	0.25-256
	Amoxycillin (25)	21	256	256	0.25-256
	Gentamicin (10)	10	256	256	0.25-256
	Tetracycline (30)	0	256	256	0.25-256
	Nalidixic acid (30)	0	>256	>256	1-256
Pseudomonas aeruginosa N = 13	Ciprofloxacin (5)	31	256	256	0.015-256
	Pefloxacin (5)	23	256	256	0.25-256
	Sparfloxacin (5)	23	256	256	0.25-256
	Ceftazidime (30)	18	32	256	0.25-256
	Ceftriazone (30)	0	16	256	0.25-256
	Augmentin (30)	0	256	256	0.25-256
	Amoxycillin (25)	0	256	256	0.25-256
	Gentamicin (10)	8	256	256	0.25-256
	Tetracycline (30)	0	256	256	0.25-256
	Nalidixic acid (30)	0	>256	>256	1-256

MIC, Minimum inhibitory concentration; MIC50/90, MIC for 50 and 90% of the organisms, respectively; ( ), content of the disc for diffusion test.

and β-lactam antibiotics inclusive. Figure 2B shows quinolone and β-lactam susceptible strains accumulate more bis-benzimidine (I8, I9, IK, SGF) while quinolone and β-lactam resistant strains which are not different from MDR accumulate less. The isolates from University College Hospital, Ibadan accumulate less drugs correlating to the fact that they are more resistant with high minimum inhibitory concentrations of quinolone compared to the rests of the hospital.

DISCUSSION

The presence of topoisomerase- (specifically mutations in *gyrA* and *parC* genes) and plasmid-mediated quinolone resistance and β-lactam genes had been used to explain in part the high level of MICs of the strains under study in Nigeria (Ogbolu et al., 2011), loss of OMPs can lower the permeability of drug into the cell and cause antimicrobial resistance (Ardanuy et al., 1998; Martinez-Martinez et al., 2000). OMPs were identified on the basis of relative mobility, the majority of the isolates lack both OmpF and OmpC. The highest MICs of fluoroquinolones and β-lactam were usually, although not always associated with those isolates lacking OMPs. The defect of the expression of OmpC and OmpF is responsible for reduced-susceptible phenotype of these strains. This result suggests that besides mutations in *gyrA*, *parC* and presence of plasmid mediated resistance loss of outer membrane, porins can also serve as the first-step mutation for developing

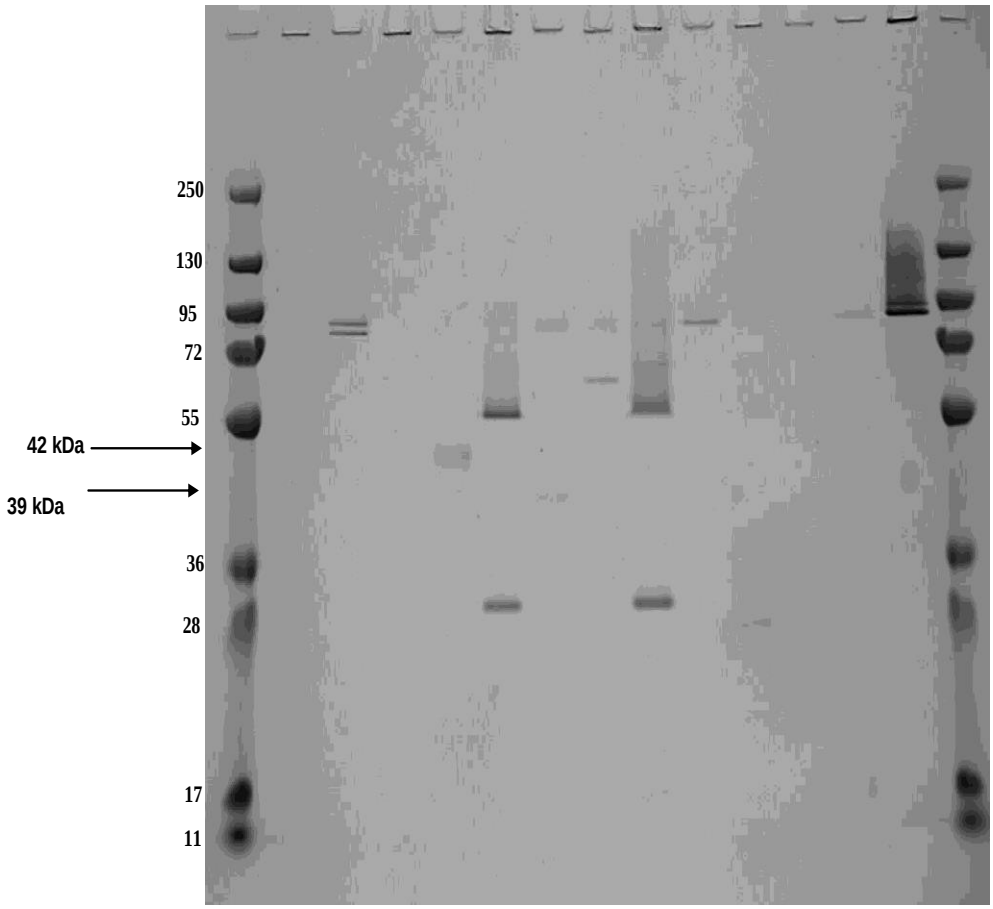

Figure 1. Example of OMP profiles of bacterial strains by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis expressing OmpC or OmpF alone or in combination. The molecular mass marker (M) and OmpC and OmpF with their corresponding sizes, 42 and 39 kDa respectively are indicated.

resistance to fluoroquinolones or β-lactam in the strains of Gram negative bacilli.

The loss of porin has been described as an important cause in the resistance between fluoroquinolones and other forms of antibiotics (Chen et al., 2003). Overall, most of the strains accumulate less bis-benzimidine than L354 wild-type control strain. This is expected since majority of the isolates are multiply drug resistant (MDR) with quinolone and β-lactam antibiotics inclusive. Quinolone susceptible strains accumulate more bis-benzimidine while quinolone resistant strains which are not different from MDR accumulate less. The isolates from University College Hospital, Ibadan accumulated fewer drugs since they were more resistant with high minimum inhibitory concentrations of quinolone compared to the rest of the hospitals. Fluoroquinolones are not subject to enzymatic degradation, so the accumulation of antibiotics within cells is determined by the relative rates of influx and efflux across the cell envelope (Everett et al., 1996).

However, drug accumulation is reduced by an active efflux system which is especially prominent in quinolone-resistant strains (Tran and Jacoby, 2002; Usui et al., 2011). Nevertheless, permeability plays a secondary role in quinolone susceptibility and resistance, in sharp contrast to the situation with β-lactam antibiotics (Nikaido, 1989). The steady-state concentration of drug taken up by the isolates was measured after 10 min of exposure to determine whether decreased accumulation may contribute to the fluoroquinolone and β-lactam resistance phenotype. Decreased activity of drugs against *E. coli* has been related to a reduced intracellular drug accumulation due to lipopolysaccharide or porin alterations impairing uptake or because of enhanced efflux (Hooper et al., 1986). Decreases in the amount of OmpF were found to be associated with decreased accumulation of fluoroquinolones in *E. coli* (Karczmarczyk et al., 2011). Similarly, Garcia-Fernandez et al. (2010) in their study, also implicated loss of porins in increased resistance to β-lactams in comparison with their respective strains, and did not discountenance the importance of the efflux mechanism as a contributor to β-lactam resistance in *K. pneumoniae.* This has also been previously shown for

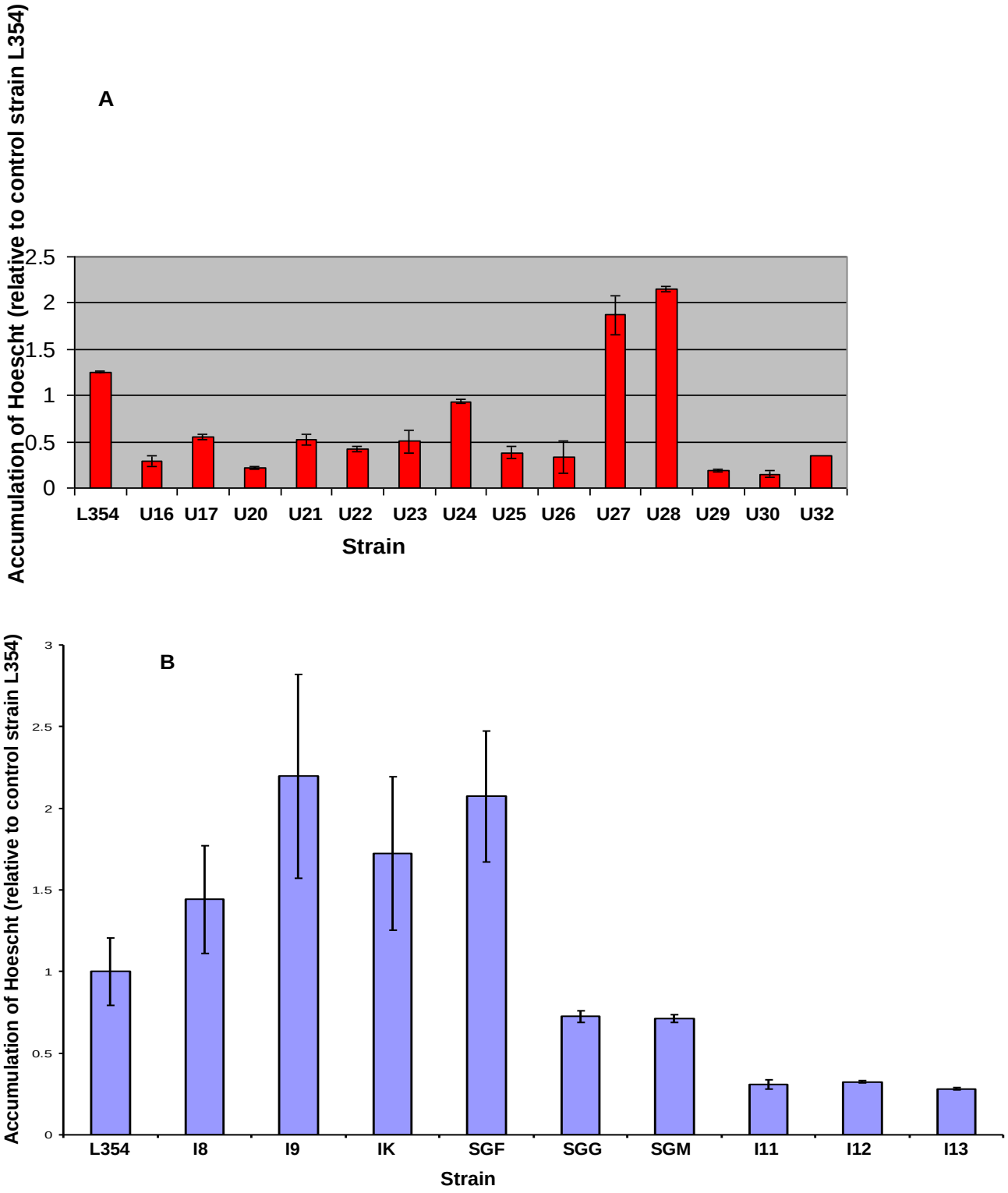

Figure 2. Accumulation of bis-benzimide by strains of Gram negative enteric bacilli. L354 is a wild type strain. (A) is for some of the strains from University College Hospital, Ibadan, while (B) is for some strains from Obafemi Awolowo University Teaching Hospital, Ile-Ife and Olabisi Onabisi Onabanjo University Teaching Hospital, Sagamu.

P. aeruginosa (Farra et al., 2008). Active efflux either singly or in tandem with OMP alterations would be responsible for the low accumulation of fluoroquinolone and β-lactam antibiotics seen in this study and their increased resistance

to both important classes of antibiotics.

Conflict of Interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

The authors thank Professor Laura Piddock (Head, Antimicrobial Research Group, Birmingham) for her support during this project. D. O. O. was the recipient of an overseas scholarship from the British Society for Antimicrobial Chemotherapy (BSAC), which supported this work.

REFERENCES

Ardanuy C, Linares J, Dominguez MA, Harnandez-Alles S, Benedi VJ, Martinez-Martinez L (1998). Outer membrane profiles of clonally related *Klebsiella pneumoniae* isolates from clinical samples and activities of cephalosporins and carbapenems. Antimicrob. Agents Chemother. 42:1636-1640.

Bedard J, Wong S, Bryan LE (1987). Accumulation of enoxacin by *Escherichia coli* and *Bacillus subtilis.* Antimicrob. Agents Chemother. 31:1348-1354.

Chapman JS, Georgopapadakou NH (1988). Routes of quinolone permeation in *Escherichia coli.* Antimicrob. Agents Chemother. 32:438-442.

Chen F, Lauderdale T, Ho M, Lo H (2003). The role of mutations in *gyrA, parC,* and *ompK*35 in fluoroquinolone resistance in *Klebsiella pneumoniae.* Microbiol. Drug Res. 9:265-271.

Clinical and Laboratory Standards Institute, CLSI (2012). Performance standards for antimicrobial susceptibility testing. M100–S22. Wayne (PA).

Everett MJ, Jin YF, Ricci V, Piddock LJV (1996). Contribution of individual mechanisms to fluoroquinolones resistance in 36 *Escherichia coli* strains isolated from humans and animals. Antimicrob. Agents Chemother. 40:2380-2386.

Farra A, Islam S, Strålfors A, Sörberg M, Wretlind B (2008). Role of outer membrane protein OprD and penicillin-binding proteins in resistance of Pseudomonas aeruginosa to imipenem and meropenem. Int. J. Antimicrob. Agents 31:427-433

Filip C, Fletcher G, Wulff JL, Earhart CF (1973). Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium lauryl sarcosinate. J. Bacteriol. 115:717-722.

Garcia-Fernandez A, Miriagou V, Papagiannitsis CC, Giordano A, Venditti M, Mancini C, Carattoli A (2010). An ertapenem-resistant extended-spectrum-beta-lactamase-producing *Klebsiella pneumoniae* clone carries a novel OmpK36 porin variant. Antimicrob. Agents Chemother. 54:4178-4184.

Hirai K, Aoyama H, Irikura T, Iyobe S, Mitsuhashi S (1986). Differences in susceptibility to quinolones of outer membrane mutants of *salmonella typhimurium* and *Escherichia coli*. Antimicrob. Agents Chemother. 29:535-538.

Hooper DC, Wolfson JS, Souza S, Tung C, McHugh GL, Swartz MN (1986). Genetic and biochemical characterization of norfloxacin resistance in *Escherichia coli.* Antimicrob. Agents Chemother. 29:639-644.

Jacoby GA (2005). Mechanisms of resistance to quinolones. Clin. Infect. Dis. 41(Suppl. 2):S120-S126.

Karczmarczyk M, Martins M, Quinn T, Leonard N, Fanning S (2011). Mechanisms of fluoroquinolone resistance in *Escherichia coli* isolates from food-producing animals. Appl. Environ. Microbiol. 77:7113-7120.

Martínez-Martínez L, Conejo MC, Pascual A, Hernández-Allés S, Ballesta S, Ramírez De Arellano-Ramos E, Benedí VJ, Perea EJ (2000). Activities of imipenem and cephalosporins against clonally related strains of *Escherichia coli* hyperproducing chromosomal beta-lactamase and showing altered porin profiles. Antimicrob. Agents Chemother. 44:2534-2536.

Nikaido H 1989). Outer membrane barrier as a mechanism of antimicrobial resistance. Antimicrob. Agents Chemother. 33:1831-1836.

Ogbolu DO, Daini OA, Ogunledun A, Alli AO, Webber MA (2011). High levels of multidrug resistance in clinical isolates of Gram-negative pathogens from Nigeria. Int. J. Antimicrob. Agents 37:62-66.

Robicsek A, Jacoby GA, Hooper DC (2006). The worldwide emergence of plasmid mediated quinolone resistance. Lancet Infect. Dis. 6:629-640.

Tran JH, Jacoby GA (2002). Mechanism of plasmid-mediated quinolone resistance. Proc. Natl. Acad. Sci. USA, 99:5638-5642.

Usui M, Uchiyama M, Baba K, Nagai H, Yamamoto Y, Asai T (2011). Contribution of enhanced efflux to reduced susceptibility of *Salmonella enterica* serovar Choleraesuis to fluoroquinolone and other antimicrobials. J. Vet. Med. Sci. 73(2):279-282.

Webber MA, Randall LP, Cooles S, Woodward MJ, Piddock LJV (2008). Triclosan resistance in *Salmonella enterica* serovar Typhimurium. J. Antimicrob. Chemother. 62:83-91.

Wolfson JS, Hooper DC (1985). The fluoroquinolones: structures, mechanisms of action and resistance, and spectra of activity *in vitro.* Antimicrob. Agents Chemother. 28:581-586.

academicJournals

Vol. 9(15), pp. 1089-1097, 15 April, 2015
DOI: 10.5897/AJMR2014.7267
Article Number: 19A178952645
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Variability in *Fusarium oxysporum* f.sp. *ciceris* causing wilt in chickpea

Amandeep Kaur[1]*, Vineet K. Sharma[1], Asmita Sirari[2], Jaspal Kaur[1], Gursahib Singh[2] and Pardeep Kumar[1]

[1]Department of Plant Pathology, Punjab Agricultural University (PAU), Ludhiana-141004, Punjab, India.
[2]Department of Plant Breeding and Genetics, Punjab Agricultural University (PAU), Ludhiana - 141004, Punjab, India.

Received 12 November, 2014; Accepted 20 March, 2015

Fusarium wilt caused by *Fusarium oxysporum* f.sp. *ciceris* (Padwick) Matuo and K. Sato is a major constraint in successful cultivation of chickpea. Therefore, in the present study, 24 isolates of *F. oxysporum* f.sp. *ciceris* collected from different chickpea growing areas of Punjab and adjoining states were assessed for morphological variations. Among 24 isolates, the maximum (8.78 mm/days) and minimum (5.00 mm/day) growth rate were exhibited by Foc-21 and Foc-15, respectively. The isolates showed growth pattern from appressed, fluffy to less fluffy and mycelial colour varied from different shades of white to purplish white. A significant variation with respect to size of micro (8.9-16.9 x 3.1-6.3 µm) and macro (21.7-64.9 x 2.7-10.0 µm) conidia was also observed. At pathogenic level, twenty isolates were studied where Foc-3, Foc-7 and Foc-22 showed virulence pattern similar to existing races 1, 2, 3 and 4 on three standard differentials *viz*. JG 62, WR 315 and L 550, whereas the rest of the isolates did not match with any of the existing race reaction. Further, the six selected genotypes could differentiate the isolates into four pathotypes based on their aggressiveness and Foc-8 was found more aggressive (98.48% wilt incidence) whereas Foc-24 was found to be least aggressive (7.22% wilt incidence). At molecular level, the sequences of internal transcribed spacers (ITS) genomic regions of isolates were studied and they showed 99% similarity with Foc sequences by basic local alignment search tool (BLAST) analysis.

Key words: *Fusarium oxysporum* f.sp. *ciceris*, wilt, chickpea, variability, differentials, pathotypes.

INTRODUCTION

Chickpea cultivation is often subjected to several biotic stresses of which diseases like *Ascochyta* blight, *Botrytis* grey mould, *Verticillium* wilt, *Sclerotinia* stem rot, dry root rot and *Fusarium* wilt are important. Among them, *Fusarium* wilt, caused by *Fusarium oxysporum* f.sp. *ciceris* (Padwick) Matuo and K. Sato has assumed serious proportions in the recent years. Throughout the world, annual chickpea yield losses due to this disease vary from 10 to 15% (Trapero-Casas and Jimenez-Diaz, 1985), but can reach even 100% under favourable

*Corresponding author. E-mail: avleensomal04@gmail.com.

Table 1. Location and source of host variety of different isolates of *F. oxysporum* f.sp. *ciceris*.

Isolate	Location of sample	Host variety
Foc-1	Dhaulakuan, HP	Channa- II
Foc-2	Shivpur, Paonta Sahib, HP	Local variety
Foc-3	Burma papri, Nahan Block, HP	Local variety
Foc-4	Hanumangarh , Rajasthan	P-12
Foc-5	Mehruwala (Dehradun), UK	Local variety
Foc-6	Ludhiana, Punjab	JG-62
Foc-7	Jaipur, Rajasthan	J-7
Foc-8	Nagaur, Rajasthan	Local variety
Foc-9	Sriganganagar, Rajasthan	Local variety
Foc-10	Dhaulakuan, HP	GPF2
Foc-11	Dhaulakuan, HP	GPF2
Foc-12	Phillaur, Punjab	Local variety
Foc-13	Phillaur, Punjab	Local variety
Foc-14	Samba , Jammu	Local variety
Foc-15	Nangal, Punjab	Local variety
Foc-16	Ludhiana, Punjab	Local variety
Foc-17	Sabhawala (Dehradun), UK	Local variety
Foc-18	Bharapur (Sirmour), HP	GPF2
Foc-19	Gurdaspur, Punjab	Local variety
Foc -20	Bikaner, Rajasthan	Local variety
Foc -21	Churu , Rajasthan	N-11
Foc -22	Dhaulakuan, HP	DKG-986
Foc -23	Sahari, Punjab	Local variety
Foc -24	RS, Gurdaspur	Local variety

Table 2. Sequence of ITS primer.

Primer	Sequence
ITS-Fu-f	5`-CAACTCCCAAACCCCTGTGA-3`
ITS-Fu-r	5`-GCGACGATTACCAGTAACGA-3`

conditions (Navas-Cortes et al., 2000). In India, yield losses estimates range from 10 – 90% every year in different regions in different cultivars (Singh and Dahiya, 1973; Jalali and Chand, 1992).

The fungus is both seed- and soil- borne and may survive in soil for up to six years even in the absence of the host (Haware et al., 1996). Considering the nature of damage and survival ability of the fungus, management of the disease is difficult either through crop rotation or application of fungicides. The most practical and cost-efficient method for management of chickpea wilt is the use of resistant varieties (Nene and Haware, 1980; Nene and Reddy, 1987; Bakhsh et al., 2007). However, evolution of new races poses a serious threat to deployment of wilt resistance in chickpea.

Therefore, regular monitoring of variation in new isolates collected from different cultivars and geographically distinct regions over the years is critical for successful resistance breeding programme. The conventional approaches to assess variation among the fungal isolates are morphological and virulence analysis. However, with the advent of DNA based molecular techniques it is now possible to assess genetic variation among the isolates. Genotyping of *F. oxysporum* f.sp. *ciceris* isolates along with virulence analysis may yield some information relevant to breeding. Keeping this in view, the present study was carried out with the objective of assessment of variability in isolates of *F. oxysporum* f.sp. *ciceris* (Foc) collected from chickpea growing areas of Punjab and adjoining states at morphological, pathogenic and molecular level.

MATERIALS AND METHODS

Collection, isolation and maintenance of isolates

A large number of isolations were made from stem and root portions of diseased chickpea plants collected from different chickpea cultivars grown in different locations in Punjab and adjoining states. The infected portion was cut into small bits, was surface sterilized in 0.1% mercuric chloride for 40-60 s followed by rinsing twice in sterilized distilled water. Later, these bits were transferred on PDA in Petri plates under complete and aseptic conditions. Plates were incubated at 25°C in BOD incubator to obtainfungal growth. Finally, a total of 24 isolates were purified and maintained on PDA slants at 4°C for further studies and designated as Foc-1, Foc-2 and so on (Table 1).

Cultural and morphological characterization

In cultural characterization, the fresh cultures were grown from 7 days old culture of each isolate separately and incubated at 25 ± 2°C. Each isolate was replicated thrice. The observations on colony diameter, colony colour, rate and pattern of growth were recorded up to 9 days at regular intervals. For morphological characterization, cultures obtained on PDA slants were examined under compound microscope (*Leica* DM 3000) using image analyzer software at 40x. The size and shape as well as septation of micro and macro conidia of each isolate were recorded.

Pathogenic characterization

For pathogenic characterization, only twenty isolates of *F. oxysporum* f.sp. *ciceris* were included in the study since the remaining four isolates seems to be morphologically similar to one or other isolates. All the isolates were subjected to the patho-genicity tests on susceptible and resistant chickpea germplasm lines (JG-62, WR-315, L-550, L-552, GL-26054 and GLK-24092). Each isolate was cultured on potato dextrose broth (PDB) for 15 days at 25 ± 2°C. Ten surface sterilized seeds of 6 differential varieties were sown in three replications in 4 x 9" poly bags containing autoclaved soil and inoculum of respective isolate. In the control, plants were sown in autoclaved soil only without inoculum. Data on disease incidence (% infected plants) were recorded at regular intervals.

Molecular characterization

All the isolates were characterized using internal transcribed spacers (ITS) primers for *Fusarium* genus and *xylanase 3* gene specific markers (Table 2).

Table 3. Cultural characteristics of different isolates of *F. oxysporum* f. sp. *Ciceris.*

Isolate	Colony diameter (mm) after inoculation		Average growth rate / day (mm)	Mycelial colour	Mycelial texture
	3rd day	9th day			
Foc 1	24.5	82.5	8.29	White	Appressed
Foc 2	31.0	78.0	6.71	Purplish	Fluffy
Foc 3	35.0	85.0	7.14	Dull white	Appressed
Foc 4	30.5	85.0	7.79	Bright white	Appressed
Foc 5	27.0	74.5	6.79	Dull white	Appressed
Foc 6	27.5	71.0	6.21	Creamish white	Less Fluffy
Foc 7	21.0	75.0	7.71	Creamish white	Appressed
Foc 8	26.0	72.0	6.57	White	Fluffy
Foc 9	25.5	71.0	6.50	Creamish white	Fluffy
Foc 10	35.0	85.0	7.14	White	Fluffy
Foc 11	29.0	80.0	7.28	White	Fluffy
Foc 12	25.5	82.5	8.14	White	Fluffy
Foc 13	25.0	72.5	6.78	White	Less Fluffy
Foc 14	22.5	64.5	6.00	Creamish white	Appressed
Foc 15	41.5	76.5	5.00	White	Appressed
Foc 16	20.0	80.0	8.57	Creamish white	Less Fluffy
Foc 17	21.0	74.5	7.64	White	Less Fluffy
Foc 18	25.5	75.5	7.14	Bright white	Appressed
Foc 19	23.5	72.5	7.00	Bright white	Appressed
Foc 20	23.5	69.0	6.50	Creamish white	Fluffy
Foc 21	23.5	85.0	8.78	Bright white	Fluffy
Foc 22	26.5	68.5	6.00	Creamish white	Less Fluffy
Foc 23	38.5	85.0	6.64	Bright white	Appressed
Foc 24	28.5	80.0	7.35	Bright white	Less Fluffy

Fungal DNA extraction from Foc isolates

The cultures from 24 Foc isolates were grown on 100 mL potato dextrose broth in 250 mL Borosil flasks and incubated at 25± 2°C in BOD incubator for fifteen days. DNA from 15 days old broth cultures of each isolate was extracted using modified CTAB extraction method (Murray and Thompson, 1980). DNA was quantified using Thermo Scientific NanoDrop™ 1000 Spectrophotometer and working dilutions were made with DNA concentration of 60 ng/ µl in each isolate.

Amplification of fungal DNA through PCR

Fungal DNA from each isolate was subjected to PCR amplification with ITS primers (ITS- Fu-f and ITS –Fu-r) specific for *Fusarium* genus (Abd- Elsalam et al., 2003). Each PCR reaction mixture of 25 µl contained 2 µl genomic DNA (60 ng/ µl), 5.0 µl 10X PCR buffer, 0.5 µl 2mM dNTPs, 1.5 µl 25 mM $MgCl_2$, 1.0 µl forward primer (20 pmol/µl), 1.0 µl reverse primer (20 pmol/ml), 0.3 µl Taq DNA polymerase (3 U/µl) and 13.7 µl nuclease free water. The PCR amplification was carried out in Eppendorf Mastercycler® pro with initial denaturation at 92°C for 1 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 0.50 s and extension at 72°C for 1 min. Final extension was carried out at 72°C for 10 min. The amplified PCR product was separated on 2% agarose gel in TBE buffer stained with good view dye (9 µl/ 150 ml buffer) and visualized in gel document system (ImagerTM1200, Alpha Innotech Corp., CA, USA).

The amplified ITS genomic region from each isolate was further sequenced by outsourcing from Xcelris Labs Ltd., Ahmedabad India and compared with other Foc sequences submitted at National Center for Boitechnology Information (NCBI).

RESULTS AND DISCUSSION

Cultural and morphological characterization

All the isolates produced well developed colonies on PDA medium with colony diameter ranging from 64.5 to 85.0 mm after 9 days of incubation. Maximum colony diameter, that is, 85.0 mm was observed in isolates Foc-3, Foc-4, Foc-10, Foc-21 and Foc-23, whereas minimum colony diameter, that is, 64.5 mm was observed in Foc-15. However, when growth rate was calculated, Foc-21 exhibited maximum growth rate of 8.78 mm/day and Foc-15 exhibited minimum growth rate of 5.0 mm/day (Table 3).

The isolates also differed in the growth pattern from appressed (Foc-1, Foc-3, Foc-4, Foc-5, Foc-7, Foc-14, Foc-15, Foc-18, Foc-19 and Foc-23), fluffy (Foc-2, Foc-8, Foc-9, Foc-10, Foc-11, Foc-12, Foc-20 and Foc-21) to less fluffy (Foc-6, Foc-13, Foc-16, Foc-17, Foc-22

Table 4. Morphological characterization of different isolates of *F. oxysporum* f.sp. *ciceris*.

Isolate	Microconidia							Macroconidia						
	Length (µm)			Width (µm)			No. of septa	Length (µm)			Width (µm)			No. of septa
	Max	Min	Mean	Max	Min	Mean		Max	Min	Mean	Max	Min	Mean	
Foc -1	21.0	12.6	16.9	8.2	4.5	6.3	0-1	-	-	-	-	-	-	-
Foc -2	16.2	10.8	13.5	6.5	3.7	5.1	0-1	29.8	18.3	24.0	7.6	4.4	6.0	1-3
Foc -3	17.1	9.9	13.5	8.1	3.0	5.5	0-1	31.7	18.9	25.3	8.3	5.0	6.6	1-4
Foc -4	13.3	9.2	11.2	5.9	4.5	5.2	0	37.4	19.8	28.6	7.0	3.1	5.0	1-3
Foc -5	13.8	7.3	10.5	6.5	3.0	4.8	0-1	77.3	52.6	64.9	8.3	4.2	6.2	1-5
Foc-6	14.6	7.4	11.0	7.1	4.7	5.9	0	70.1	42.6	56.2	13.4	6.6	10.0	1-3
Foc -7	12.1	6.1	9.1	5.0	3.4	4.2	0-1	72.3	50.1	61.2	7.1	3.1	5.1	1-5
Foc-8	17.1	11.0	14.1	7.0	2.7	4.8	0-1	-	-	-	-	-	-	-
Foc-9	15.5	10.2	12.8	5.2	3.8	4.5	0-1	-	-	-	-	-	-	-
Foc-10	12.2	5.7	8.9	4.2	2.1	3.1	0	-	-	-	-	-	-	-
Foc -11	-	-	-	-	-	-	-	61.4	29.1	45.2	8.3	4.0	6.1	1-5
Foc-12	15.8	9.8	12.8	6.9	5.2	6.0	0-1	-	-	-	-	-	-	-
Foc-13	-	-	-	-	-	-	0	60.6	40.7	50.6	5.3	3.2	4.2	1-2
Foc-14	16.6	10.7	13.6	5.1	3.2	4.1	0-1	30.9	17.9	24.4	4.2	2.1	3.1	1-2
Foc-15	16.1	7.8	11.9	4.8	2.0	3.4	0	29.0	17.9	23.4	4.3	2.7	3.5	1-3
Foc-16	13.2	10.2	11.7	6.9	5.0	5.9	0-1	39.0	22.5	30.7	4.3	3.3	3.8	1-3
Foc-17	-	-	-	-	-	-	-	78.7	21.1	49.9	3.1	2.3	2.7	1-3
Foc-18	10.7	8.6	9.6	4.4	3.0	3.7	0	47.1	20.4	33.7	6.2	4.5	5.3	1-3
Foc-19	16.7	12.1	14.4	6.9	2.7	4.8	0	-	-	-	-	-	-	-
Foc-20	-	-	-	-	-	-	-	27.0	16.5	21.7	3.6	2.6	3.1	1-3
Foc-21	15.3	12.2	13.7	4.9	2.9	3.9	0-1	-	-	-	-	-	-	-
Foc-22	17.5	10.8	14.1	6.0	2.4	4.2	0	35.2	20.3	27.7	5.1	3.5	4.3	1-3
Foc-23	13.0	9.7	11.3	5.7	3.4	4.5	0	-	-	-	-	-	-	-
Foc-24	18.0	10.7	14.3	6.1	4.1	5.1	0-1	60.0	21.8	40.9	4.5	3.0	3.7	1-3
CD at 5 %	0.62	0.55		0.40	0.36			2.36	1.28		0.44	0.47		

(-) Not formed; Max- maximum; Min- minimum.

and Foc-24). The cultures of different isolates also exhibited variation in mycelial colour which ranged from different shades of white to purplish. Isolates Foc-4, Foc-18, Foc-19, Foc-21, Foc-23 and Foc-24 showed bright white mycelial colour, whereas, Foc-6, Foc-7, Foc-9, Foc-14, Foc-16, Foc-20 and Foc-22 showed creamish white mycelial colour. White mycelial colour was produced by isolates Foc-1, Foc-8, Foc-10, Foc-11, Foc-12, Foc-13, Foc-15 and Foc-17. Two isolates Foc-3 and Foc-5 produced dull white mycelial colour, whereas one isolate Foc-2 produced purplish mycelial colour. Flat to fluffy, white to pinkish mycelial growth has been previously observed by other workers (Patil et al., 2005; Barhate et al., 2006). Singh et al. (2010) also observed dull white to pinkish white, thin and flat hairy to fluffy growth with irregular margins. The isolates showed considerable variation with respect to size of micro and macro conidia (Table 4). Size of microconidia varied from 5.7-21.0 x 2.00 – 8.2 µm with 0-1 septa, whereas size of macroconidia varied from 16.5-78.7 x 2.1- 13.4 µm with 1-5 septa. Similarly, average length and width of microconidia showed considerable variation (8.9-16.9 x 3.1-6.3 µm) in different isolates of *F. oxysporum* f.sp. *ciceris*. Foc-1 produced largest microconidia having maximum mean length and width (16.9 x 6.3 µm), whereas Foc-10 produced smallest microconidia having minimum mean length and width (8.9 x 3.1 µm). Likewise, average size of macroconidia also varied considerably (21.7-64.9 x 2.7-10.00 µm). Largest macroconidia were produced by Foc-6 with mean size of 56.2 x 10.00 µm, whereas smallest macroconidia were produced by Foc-20 with mean size of 3.1-21.7 µm.

Pathogenic characterization

On a set of six chickpea genotypes *viz*., GL 26054, JG 62, WR 315, GLK 24092, L 550 and L 552, Foc-8 was found more aggressive, causing maximum average wilt incidence (98.48%), whereas Foc-24 was found least aggressive producing minimum average wilt incidence (7.22%) (Table 5). However, Foc-3, Foc-7 and Foc-22

Table 5. Percent wilt incidence induced by different Foc isolates on selected chickpea genotypes.

Isolate/genotype	Wilt incidence (%)						
	GLK 24092	L 550	JG 62	WR 315	L 552	GL 26054	Average
Foc-1	92.31	70.00	83.33	85.71	0.00	50.00	63.56
Foc-2	100.00	92.31	85.71	75.00	100.00	0.00	75.50
Foc-3	90.00	58.33	53.85	0.00	60.00	66.67	54.81
Foc-4	92.86	84.62	88.89	83.33	100.00	75.00	87.45
Foc-5	53.85	53.33	25.00	62.50	0.00	100.00	58.94
Foc-7	69.23	50.00	41.67	0.00	100.00	0.00	43.48
Foc-8	90.91	100.00	100.00	100.00	100.00	100.00	98.48
Foc-9	86.67	69.23	84.62	66.67	0.00	100.00	81.44
Foc-10	100.00	81.25	100.00	77.78	100.00	100.00	93.17
Foc-11	66.67	85.71	15.38	22.22	0.00	50.00	48.00
Foc-13	77.78	68.75	100.00	100.00	100.00	33.33	79.90
Foc-14	92.31	88.89	66.67	100.00	66.67	75.00	81.59
Foc-15	0.00	25.00	16.67	0.00	0.00	0.00	8.33
Foc-16	75.00	72.73	77.78	66.67	100.00	100.00	82.03
Foc-17	69.23	70.00	0.00	0.00	0.00	50.00	37.85
Foc-18	100.00	81.25	100.00	60.00	100.00	50.00	81.88
Foc-20	33.33	50.00	16.67	0.00	100.00	33.33	38.89
Foc-22	42.86	45.45	37.50	0.00	0.00	10.00	27.16
Foc-23	93.33	70.00	55.56	75.00	100.00	100.00	82.31
Foc-24	0.00	33.33	0.00	10.00	0.00	0.00	7.22

Table 6. Grouping of different isolates as different pathotypes.

Pathotype	Isolate
I	Foc-4, Foc-8, Foc-9, Foc-10, Foc-14, Foc-16, Foc-18, Foc-23.
II	Foc-1, Foc-2, Foc-3, Foc-5, Foc-7, Foc-11, Foc-13
III	Foc-17, Foc-20, Foc-22
IV	Foc-24, Foc-15

Table 7. Grouping based on pathogenic variation.

Group	Sub group	Isolate
1	1a	Foc-1, Foc-9, Foc-5, Foc-11 and Foc-17
	1b	Foc-22, Foc-15, Foc-24, Foc-3, Foc-7 and Foc-20
2	2a	Foc-2, Foc-13 and Foc-18
	2b	Foc-14, Foc-16, Foc-23, Foc-8, Foc-4 and Foc-10

behaved like existing in races 1, 2, 3 and 4 on three genotypes JG 62, WR 315 and L 550. Rest of the isolates differed in their virulence behavior on these three genotypes. Among these isolates, it was observed that Foc-17 and Foc- 24 were avirulent on highly susceptible variety JG 62, whereas Foc- 1, Foc-2, Foc-4, Foc-5, Foc-8, Foc-9, Foc-10, Foc-11, Foc-13, Foc-14, Foc-16, Foc-18, Foc-23 and Foc-24 were virulent on highly resistant variety WR 315.

The six selected genotypes could differentiate the level of aggressiveness of all the isolates, thus giving them the status of pathotypes. Therefore, on the basis of mean aggressiveness, the 20 isolates could be converted into four pathotypes as given in Table 6. The isolates were also differentiated on the basis of their virulence reaction on each chickpea genotype, using DARwin software. The isolates were converted into two groups (Table 7). The first group consisted of two sub groups, that is, 1a (Foc-1,

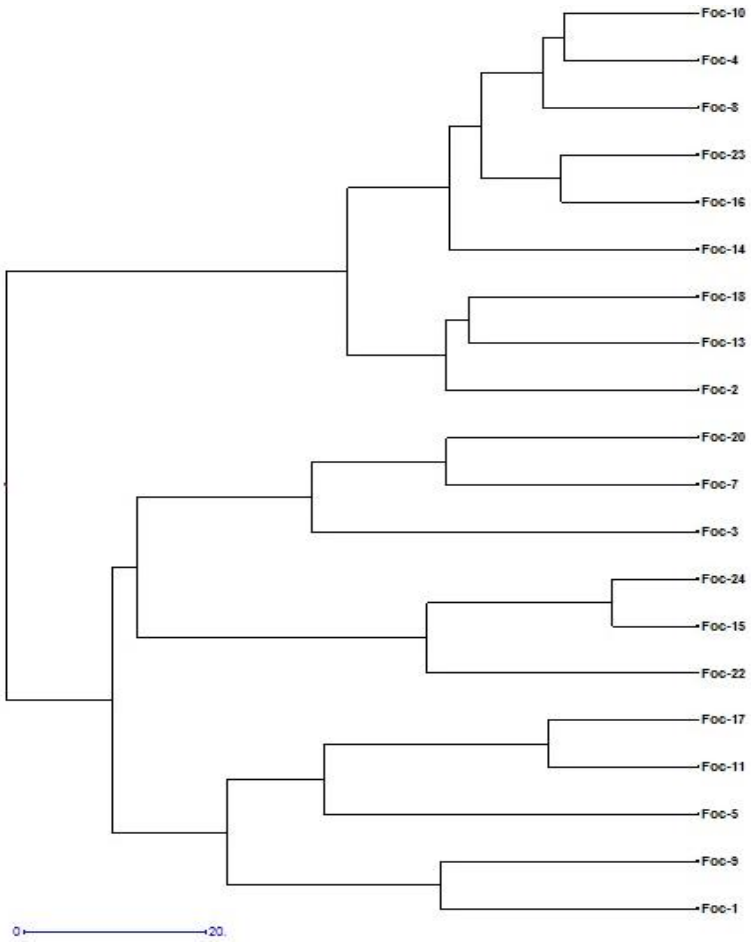

Figure 1. Dendrogram based on pathogenic variation among Foc isolates.

Foc-9, Foc-5, Foc-11 and Foc-17) and 1b (Foc-22, Foc-15, Foc-24, Foc-3, Foc-7 and Foc-20). Similarly, the second group also consisted of two sub groups, that is, 2a (Foc-2, Foc-13 and Foc-18) and 2b (Foc-14, Foc-16, Foc-23, Foc-8, Foc-4 and Foc-10) (Figure 1). The same standard differential was followed by several workers and existence of four races *viz;* race 1, 2, 3 and 4 was reported (Bayraktar and Dolar, 2012; Mandhare et al., 2011; Barhate and Dake, 2006). Recently, a new race (race 6) was reported to occur in India by Sharma et al. (2014).

Molecular characterization

Fusarium genus specific 18 S ribosomal DNA based ITS markers were used for true identification and study of genetic variation among Foc isolates. The ITS primers amplified a region of ~400 bp size from genomic DNA of all isolates (Figures 2 and 3). Further, amplicons were eluted using gel extraction kit, purified and sequenced by outsourcing from Xcelris Labs Ltd., Ahmedabad India. The sequences were aligned using DNA baser software and compared with other Foc sequences from standard database GenBank. Nineteen isolates were further subjected to Basic Local Alignment Search Tool (BLAST) software to compare the sequences of Foc, previously submitted at National Center for Biotechnology Information (NCBI) data base. All sequences resembled 99% similarity with Foc sequences. Dendrogram on the basis of sequence homologies among all the isolates was also generated by using dendroscope and it was found that 20 isolates were further grouped into three major groups (Figure 6). First major group consisted of 17 isolates Foc-13, Foc-14, Foc-3, Foc-2, Foc-4, Foc-15, Foc-17, Foc-23, Foc-11, Foc-8, Foc-20, Foc-5, Foc-22, Foc-1, Foc-24, Foc-18 and Foc-16. The other two groups consisted of Foc-9 and Foc-10. The ITS region sequence of Foc-15 have been deposited at NCBI, with Accession no. KM253762.

In the present study, presence of 700 bp fragment amplified by xylanase 3 gene (Figures 4 and 5) in all the isolates confirmed absence of race 4 in any of the isolates. Foc race 4 was distinguished with xylanase 3 gene by absence of amplification product only in this

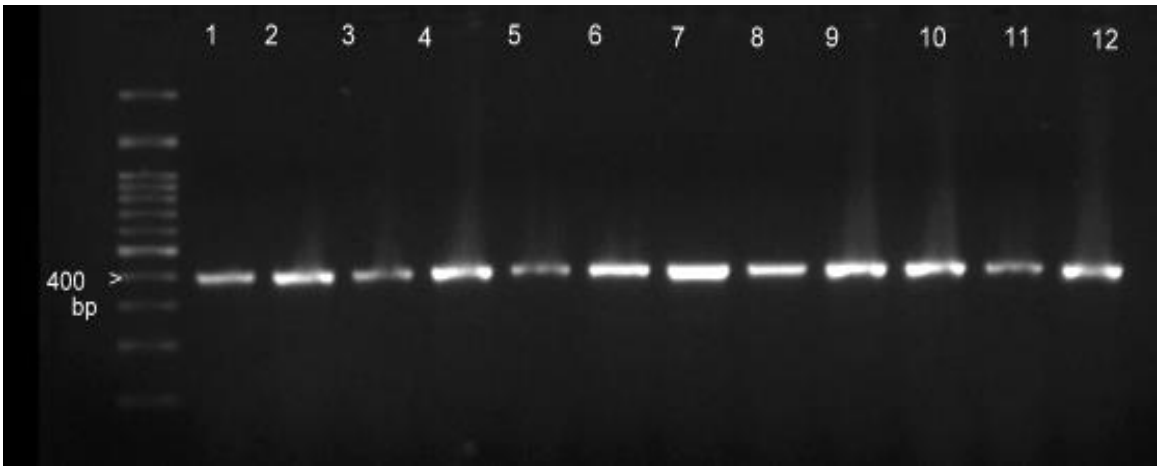

Figure 2. PCR amplification of genomic DNA of Foc isolates (1-12) with ITS markers.

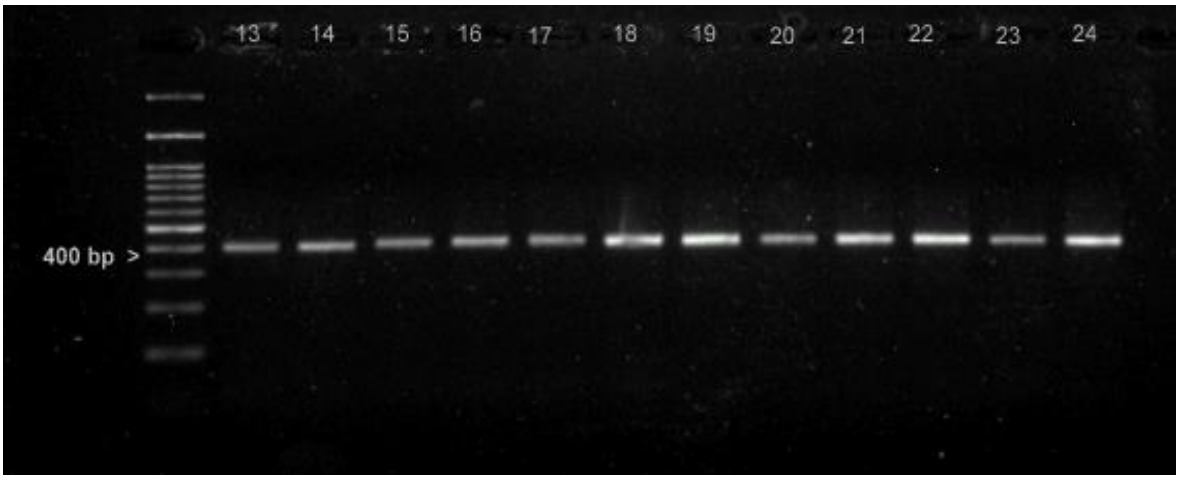

Figure 3. PCR amplification of genomic DNA of Foc isolates (13-24) with ITS markers.

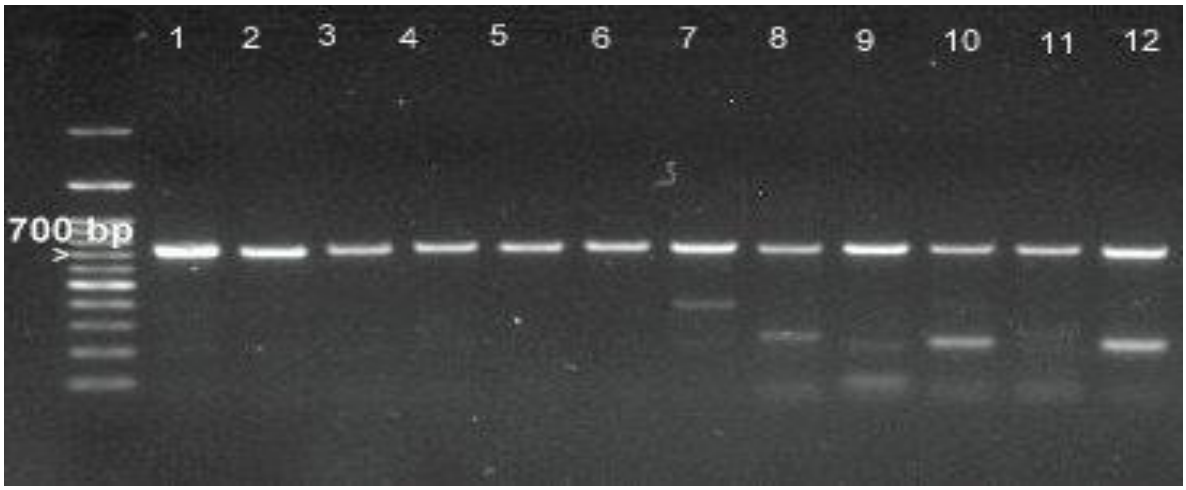

Figure 4. PCR amplification of genomic DNA of Foc isolates (1-12) with *Xylanase 3* gene specific marker

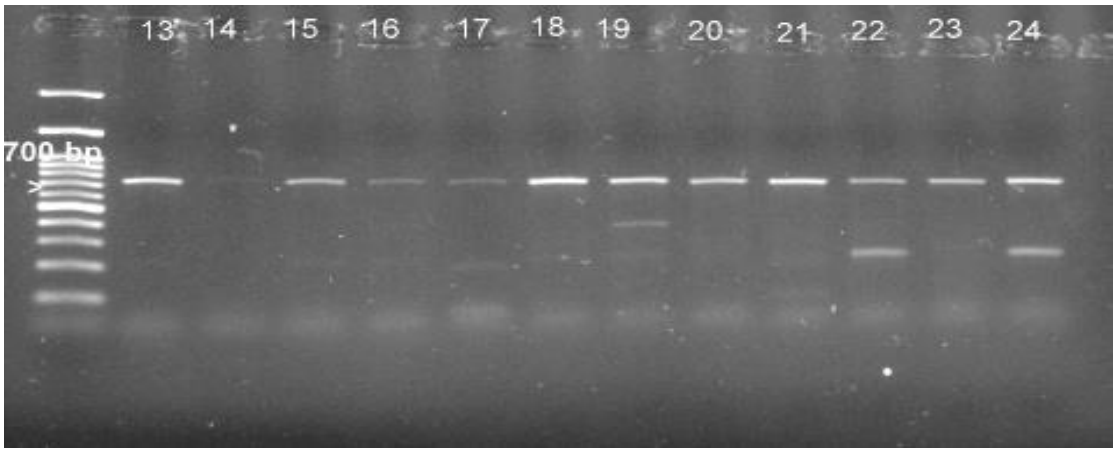

Figure 5. PCR amplification of genomic DNA of Foc isolates (13-24) with *Xylanase 3* gene specific marker.

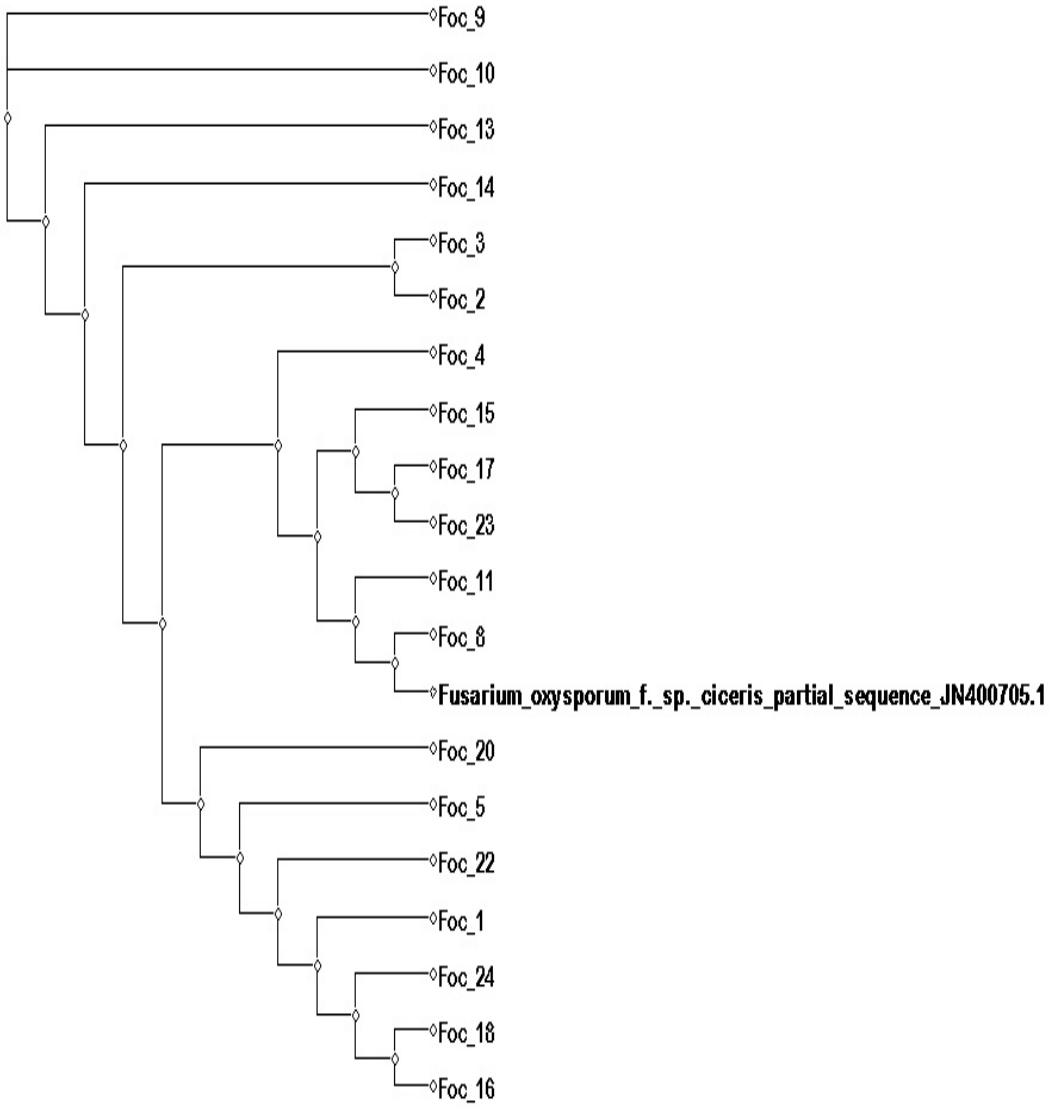

Figure 6. Maximum-likelihood phylogenetic tree based on the alignment of the partial sequences.

race (Gurjar et al., 2009). Any correlation between geographical region and virulence variation with genetic diversity was not observed in the case of both markers (Singh et al., 2006; Sharma et al., 2009; Mandhare et al., 2011; Sivaramakrishnan et al., 2002). In a similar study by Kelly et al. (1994), it was observed that RAPD marker analysis converged the Foc isolates into two groups, yellowing type and wilt syndrome type. RAPDs markers have also been developed into SCAR markers specific to Foc (Durai et al., 2012).

Dubey et al. (2010) observed high level of genetic diversity using ITS-RFLP analysis of Foc isolates, which converged the isolates into six groups. Recently, Sharma et al. (2014) reported occurrence of race 6 in India, which is of Mediterranean and USA region. They also reported application of DArT markers in assessment of genetic diversity in Foc pathogen, with few race specific unique alleles.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

Bakhsh A, Iqbal SM, Haq IK (2007). Evaluation of chickpea germplasm for wilt resistance. Pak. J. Bot. 39:583-93.

Barhate BG, Dake GN (2006). Variability for virulence in *Fusarium oxysporum* f.sp. *ciceris* causing wilt of chickpea. Legume Res. 29:308-10.

Barhate BG, Dake GN, Game BC, Bachkar CB (2006). Sources of resistance to Fusarium wilt of chickpea. Legume Res. 29:232-32.

Bayraktar H, Dolar F (2012). Pathogenic variability of *Fusarium oxysporum* f.sp.*ciceris* isolates from chickpea in Turkey. Pak. J. Bot. 44:821-28.

Durai M, Dubey SC, Tripathi A (2012). Genetic diversity analysis and development of SCAR marker for detection of Indian populations of *Fusarium oxysporum* f. sp. *ciceris* causing chickpea wilt. Folia Microbiol. 57:229-35.

Gurjar G, Barve M, Giri A, Gupta V (2009). Identification of Indian pathogenic races of *Fusarium oxysporum* f. sp. *ciceris* with gene specific, ITS and random markers. Mycologia 101:484-95.

Haware MP, Nene YL, Natarajan M (1996). Survival of *Fusarium oxysporum* f.sp. *ciceri*. Plant Dis. 66:809-10.

Jalali BL, Chand H (1992). Plant Diseases of International Importance. Diseases of Cereals and Pulses. Prentice Hall, Englewood Cliffs N J. Vol 1. pp. 429-44.

Kelly, Alcala-Jimenez AR, Bainbridge BW, Heale JB, Perez-Artes E, Jimenenz-Diaz RM (1994). Use of genetic fingerprinting and random amplified polymorphic DNA to characterize pathotypes of *Fusarium oxysporum* f.sp. *ciceris* infecting chickpea. Phytopathology 84:1293-98.

Mandhare VK, Deshmuk GP, Patel JV, Kale AA, Chavan UD (2011). Morphological, pathological and molecular characterization of *Fusarium oxysporum* f.sp. *ciceris* isolates from Maharashtra, India. Indo. J. Agric. Sci. 12:47-56.

Murray MG, Thompson WF (1980). Rapid isolation of high molecular weight DNA. Nucleic Acids Res. 8:4321-25.

Navas-Cortes JA, Hau B, Jimenez-Diaz RM (2000). Yield loss in chickpea in relation to development of Fusarium wilt epidemics. Phytopathology 90:1269-78.

Nene YL, Haware MP (1980). Screening chickpea for resistance to wilt. Plant Dis. 64:379-80.

Nene YL, Reddy MV (1987). Chickpea diseases and their control. The Chickpea CAB International, Oxon, UK. pp. 233-70.

Patil PD, Mehetre SS, Mandare VK, Dake GN (2005). Pathogenic variation among *Fusarium* isolates associated with wilt of chick-pea (*Cicer arietinum* L.). Ann. Plant Prot. Sci. 13:427-30.

Sharma M, Kumar RV, Narayan JR, Kannan S, Hoisington D, Pande S (2009). Genetic diversity in Indian isolates of *Fusarium oxysporum* f.sp. *ciceris,* chickpea wilt pathogen. Afr. J. Biotechnol. 8:1016-23.

Sharma M, Nagavardhini A, Thudi M, Ghosh R, Pande S, Varshney RK (2014). Development of DArT markers and assessment in *Fusarium oxysporum* f.sp. *ciceris,* wilt pathogen of chickpea (*Cicer arietinum* L.). BMC Genomics 15:454.

Singh BP, Saikia R, Yadav M, Singh R, Chauhan VS, Arora D (2006). Molecular characterization of *Fusarium oxysporum* f.sp. *ciceris* causing wilt of chickpea. Afr. J. Biotechnol. 5:497-502.

Singh KB, Dahiya BS (1973). Breeding for wilt resistance in chickpea. Problem and Breeding for wilt Resistance in Bengal gram Symp. Indian Agricultural Research Institute, New Delhi. pp. 13-14.

Singh RK, Hasan Abul, Chaudhary RG (2010). Variability in *Fusarium oxysporum* f.sp. *ciceris* causing wilt in chickpea. Arch. Phytopathol. Plant Prot. 43:987- 95.

Sivaramakrishnan S, Kannan Seetha, Singh SD (2002). Genetic variability of Fusarium wilt pathogen isolates of chickpea assessed by molecular markers. Mycopathology 155:171-78.

Trapero-Casas A, Jimenez-Diaz RM (1985). Fungal wilt and root rot diseases of chickpea in southern Spain. Phytopathology 75:1146-1151.

academicJournals

Vol. 9(15), pp. 1098-1104, 15 April, 2015
DOI: 10.5897/AJMR2015.7445
Article Number: DF4453A52647
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Exploiting novel rhizosphere *Bacillus* species to suppress the root rot and wilt pathogens of chickpea

Kodoth Padinhare Smitha*, Rajeswari Mohan, Alice Devadason and Thiruvengadam Raguchander

Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, India.

Received 23 February, 2015; Accepted 13 April, 2015

Thirty isolates of *Bacillus* were collected from chickpea rhizosphere and screened for their *in vitro* inhibition against root rot (*Rhizoctonia bataticola*) and wilt (*Fusarium oxysporum* f.sp. *ciceri)* pathogens and growth promotion of chickpea. Based on the *in vitro* inhibition and growth promotion tests, the best eight isolates were selected and PCR-based detection of antibiotics genes *viz.*, surfactin, iturin, fengycin and bacillomycin D was carried out. The isolate which produced all these antibiotics and showed maximum *in vitro* inhibition (CaB 5) was further used for crude antibiotics extraction and inhibition assays. The presence of antibiotics in crude extract was detected through TLC. The inhibitory effect of the crude extract was proved through agar-well diffusion assay and spore germination inhibition test. From this study, it was inferred that the *Bacillus subtilis* strain CaB5 was promising in inhibiting the root rot and wilt pathogens of chickpea and enhance seedling vigour.

Key words: Biological control, plant growth promotion, surfactin, iturin, fengycin.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the world's third most important food legume with 96% of the total cultivated area in the developing countries. It is the premier pulse crop of Indian subcontinent. India is the largest producer as well as consumer of chickpea in the world. About 65% of the global chickpea area falls in India, corresponding to 68% of the global production (FAOSTAT, 2012). In India, chickpea is grown in an area of 8.3 Mha with a production of 7.7 MT and productivity of 928 kg/ha (Directorate of Economics and Statistics, 2011-12). Despite its economic importance, the productivity is low owing to many biotic and abiotic stresses. Chickpea is affected by many soil-borne diseases of which the root rot pathogen *Rhizoctonia bataticola* (Taub). Butler and vascular wilt fungus *Fusarium oxysporum* f.sp. *ciceri* are the most important in Indian subcontinent. At field level, these diseases can be managed to some extent by using seed dressing fungicides and chemical sprays. Of late, there has been a shift from chemical to biological control methods owing to the toxicity hazards and environmental pollution due to overuse of chemicals.

Bacillus species are outstanding biocontrol agents as they show effective root colonization, multiple modes of action and promising ability to sporulate (Kloepper et al.,

*Corresponding author. E-mail: smitpath@gmail.com.

2004). Cyclic lipopeptides of the surfactin, iturin and fengycin families are important metabolites produced by *Bacillus* species. *Bacillus subtilis* has an average of 4-5% of its genome devoted to antibiotic synthesis and potential to produce more than two dozen of structurally diverse antimicrobial compounds (Stein, 2005). Turner and Backman (1991) found that *Bacillus* sp. colonized the root surface, increased plant growth and caused lysis of fungal mycelia. Their endospore-forming ability also makes these bacteria one of the best candidates for developing efficient biopesticide products from a techno-logical point of view (Gordillo and Maldonado, 2012). Hence an effort was made to assess the *in vitro* efficacy of *Bacillus* spp. against root rot and wilt pathogens of chickpea.

MATERIALS AND METHODS

Isolation of the pathogens

Chickpea plants showing typical symptoms of root rot and wilt were collected from the fields and used for isolation of the pathogens. Isolation was made from collar and stem regions in the case of *Fusarium oxysporum f.sp. ciceri* and root and collar regions were used for isolating *Rhizoctonia bataticola*. The tissues were washed in running tap water, cut into small bits of 5-10 mm, surface sterilized with 0.1% mercuric chloride for 30 s, blotted dried on sterile filter paper and plated on potato dextrose agar (PDA) medium. The plates were incubated at 22-25°C for 2-3 days and actively growing mycelia transferred to PDA slants by hyphal tip method. The pathogenicity of both *F. o. f.sp. ciceri* and *R. bataticola* were proved as per Koch's postulates.

Collection of *Bacillus* strains

A survey was conducted in the major chickpea growing areas of Tamil Nadu *viz*., Coimbatore, Tirupur and Dindigul during the period from October to December 2013. Rhizosphere soil samples were collected and *Bacillus* spp. were isolated by serial dilution plate technique. Ten grams of soil was added to 90 ml of sterile distilled water and subjected to 80°C for 20 min in water bath so that only the spores of bacteria remained in the suspension. This was serially diluted up to 10^{-7} and plated on nutrient agar (NA) plates. These isolates were purified and maintained on NA slants.

In vitro evaluation of *Bacillus* strains

Thirty rhizosphere *Bacillus* strains were tested for their *in vitro* efficacy against *F. o. f.sp. ciceri* and *R. bataticola* by dual culture technique (Dennis and Webster, 1971). The bacterial culture was streaked at one side of 90 mm Petri dish (1 cm from the edge of a plate) with PDA medium and mycelial disc (5 mm diameter) of actively growing (seven days old) culture of the pathogens placed on the opposite side in the Petri dish perpendicular to the bacterial streak. The experiment was laid out in completely randomised design with three replications for each treatment. The plates were incubated at room temperature (28 ± 2°C) for seven days and the mycelial inhibition of pathogen over control was calculated according to the formula given by Vincent (1947).

PI = (C-T) / C x 100

Where, PI– Inhibition percentage, C– Rate of growth of pathogen in control and T– Rate of growth of pathogen in treatment

Plant growth promotion tests

The eight bacterial isolates which showed *in vitro* inhibition of the pathogens were inoculated in a conical flask with 100 ml of nutrient broth. Required quantity of chickpea seeds (cv. CO4) were soaked in bacterial suspension containing 3 x 10^8 cfu ml^{-1} for 2 h and shade dried. The seeds soaked in sterile water served as control. Plant growth-promoting activity of rhizospheric bacterial isolates was assessed based on the seedling vigour index by the standard roll towel method (ISTA, 1993). Ten seeds were kept over the pre-soaked germination paper. The seeds were held in position by placing another pre-soaked germination paper and gently pressed. The polythene sheet along with seeds was then rolled and incubated in growth chamber for 10 days. Three replications were maintained for each treatment. The root and shoot length of individual seedlings were measured and the germination percentage of seeds were calculated.

Plant growth promotion was also tested by pot culture method. Bacterized seeds were sown in pots. Fifteen seeds were maintained for each treatment. The root and shoot length of individual seedlings were measured and germination percentage of seeds was calculated. The vigour index was calculated by using the formula as described by Baki and Anderson (1973).

Vigour index = per cent germination x seedling length (shoot length + root length)

Detection of antibiotic genes of *Bacillus* strains

To confirm that the selected strains have the capacity to produce antibiotics, the genomic DNA was isolated for antibiotic gene detection through PCR. Genomic DNA was isolated using the cetyltrimethyl ammonium bromide (CTAB) method described by Knapp and Chandlee (1996), with slight modifications (Melody, 1997). The antibiotic genes surfactin, iturin, fengycin and bacillomycin D were amplified using the primers described below.

Surfactin

The forward primer SUR3F (5'ACAGTATGGAGGCATGGTC 3') and reverse primer SUR3R (5' TTCCGCCACTTTTTCAGTTT 3') were used for amplification of surfactin gene (440 bp) (Ramarathnam, 2007). PCR amplification was performed in a thermocycler using the following conditions: Initial denaturation at 94°C for 3 min, 40 cycles consisting of 94°C for 1 min (denaturation), 57°C for 1 min (annealing), 72°C for 1min (primer extension) and final extension at 72°C for 10 min.

Iturin A

The forward primer ITUD1F (5' GATGCGATCTCCTTGGATGT 3') and reverse primer ITUD1R (5' ATCGTCATGTGCTGCTTGAG 3') were used for amplification of iturin A gene (648 bp) (Ramarathnam, 2007). The PCR reaction conditions were initial denaturation at 94°C for 3 min, 40 cycles consisting of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension at 72°C for 10 min.

Fengycin D

The forward primer FEND1F (5'TTTGGCAGCAGGAGAAGTTT3') and

reverse primer FEND1 R (5'GCTGTCCGTTCTGCTTTTTC3') were used for amplification of fengycin gene (964 bp) (Athukorala et al., 2009). PCR amplification conditions used were as follows: Initial denaturation at 94°C for 3 min, 40 cycles consisting of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 1 min (primer extension) and final extension of 72°C for 10 min.

Bacillomycin D

The forward primer BACC1F (5'GAAGGACACGGCAGAGAGTC3') and reverse primer BACC1R (5'CGCTGATGACTGTTCATGCT3') (Operon, Inc., Alameda, CA) were used for amplification of bacillomycin D gene (876 bp) (Ramarathnam, 2007).

PCR amplification conditions were initial denaturation at 94°C for 3 min, 40 cycles consisting of 94°C for 1 min (denaturation), 62°C for 1 min (annealing), 72°C for 1 min (primer extension) and a final extension of 72°C for 10 min.

Agarose gel electrophoresis and gel documentation

Agarose gel electrophoresis was performed based on the method given by Sambrook et al. (1999) to check the quality of DNA and also to separate the products amplified through the polymerase chain reaction. After separation on 1.5% agarose gel at 50 UV, the PCR products were stained with ethidium bromide (0.5 µg/ml), photographed and analyzed using a gel documentation system.

Extraction of crude antibiotics

Based on the presence of antibiotic genes, *in vitro* inhibition and growth promotion, the best isolate was selected for extraction of crude antibiotics. The crude antibiotics were extracted as per the method described by McKeen et al. (1985). The bacteria was grown in Landy's medium (20 g D-glucose, 5 g L-glutamic acid, 1.02 g $MgSO_4 \cdot 7H_2O$, 1 g KH_2PO_4, 0.5 g KCl and 1 ml of trace element solution (0.5 g $MnSO_4 \cdot 4\ H_2O$, 0.16 g $CuSO_4 \cdot 5H_2O$, and 0.015 g $FeSO_4 \cdot 7H_2O$ in 100 ml of water per litre). The pH of the media was adjusted to 6.0 to 6.2 with 5 N NaOH. A loopfull of 24-h old bacterial culture was inoculated into 100 ml of Landy medium and the inoculated flasks were incubated on a shaker at 170 rpm and 30°C for 3 days. The production medium was centrifuged for 20 min at 12,000 rpm to remove bacterial cells. The antibiotics were precipitated from the supernatant by adjusting the pH to 2.5 with concentrated HCl. This was centrifuged for 10 min at 12,000 rpm. The pellet containing the active fraction was extracted thrice with 80% ethanol. The ethanol extract was dried under vacuum at 55°C on a rotary evaporator. Inactive substances were removed by sequential extraction with ethyl acetate and acetone. The resulting residue was dissolved in 80% ethanol and stored at 4°C.

Thin layer chromatography (TLC) of crude antibiotics

The 80% ethanol fraction with antibiotic activity was spotted onto 20 × 20-cm silica gel plates. The plates were developed with ethanol : water (2:1, v/v) and the bands were visualized with UV light and also by spraying 0.2 g of ninhydrin per 100 ml of 95% ethanol and heated at 110°C for 5-10 min to detect ninhydrin positive materials.

***In vitro* assay using crude antibiotics**

The efficacy of the crude antibiotics in pathogen inhibition was tested by agar well diffusion method and by cavity slide technique.

Mycelial growth inhibition assay

The agar well diffusion assay, as reported by Tagg and McGiven (1971) and modified by Islam et al. (2012) was used to determine the antagonistic activity of crude antibiotic extract. PDA medium was poured into each sterile Petri dish, followed by placement of 5 mm diameter mycelial disc of the pathogen at the centre of the plates. A 7 mm diameter well was made by punching the agar with a sterile cork borer on the corner of the plate in four places with equal distance. Then the crude antibiotic extract from the selected isolate CaB5 was poured into the wells at the rate of 50 µl per well and incubated for 96 h at 28±2°C. The inhibitory activity was expressed as the percent growth inhibition, as compared to the control with solvent alone, according to the following formula:

Growth inhibition (%) = (DC − DT)/DC × 100.

where, DC, diameter of fungal colony in control; and DT, diameter of fungal colony with treatment (Pandey et al., 1982).

Spore germination inhibition assay

Effect of crude antibiotics on conidial germination was tested by cavity slide technique. One drop of the culture filtrate of the isolate *B. subtilis* CaB5 was placed in a sterile cavity slide and allowed to air dry. One drop of the conidial suspension of the pathogen (5×10^4 conidia/ml) was added and mixed thoroughly. Conidial suspension + sterile distilled water served as control. The slides were kept in moist growth chamber and incubated at 25±2°C. Observation on conidial germination of pathogen was recorded at 6, 24, and 36 h after incubation by microscopic examination.

Statistical analysis

The data were statistically analyzed (Rangasamy, 1995) using the IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines (Gomez and Gomez, 1984). The percentage values of the *in vitro* inhibition were arcsine transformed. Data were subjected to the analysis of variance (ANOVA) at two significant levels (< 0.05 and < 0.01) and means were compared by Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

Biological control using antagonists provides an alternative to chemicals in plant disease management. The myco-parasitic potential of *Bacillus* spp. is well documented (Johri et al., 2003; Saharan and Nehra, 2011). In the present study, the initial screening of 30 isolates of *Bacillus* collected from different chickpea rhizosphere soils resulted in the selection of eight isolates which showed inhibition of both root rot (*R. bataticola)* and wilt (*F. oxysporum* f.sp. *ciceri*) pathogens of chickpea under laboratory conditions. The *Bacillus* genus specific primers BCF1 (CGGGAGGCAGCAGTAGGGAAT) and BCR2 (CTCCCCAGGCGGAGTGCTTAAT) amplified a fragment of approximately 546 bp corresponding to the region of the 16S-23S rRNA intervening sequence for *Bacillus* sp. Among the eight isolates, the *Bacillus* strain CaB5 (*Cicer arietinum Bacillus* 5) showed 53.3% inhibition of *F. oxysporum* f.sp. *ciceri* and 58.5% inhibition of

Table 1. *In vitro* antagonistic activity of *Bacillus* isolates against *F. oxysporum* f. sp. *ciceri* and *Rhizoctonia bataticola*.

Bacillus isolate	Percent inhibition over control of *F. oxysporum f.*sp. *ciceri*	Percent inhibition over control of *R. bataticola*
CaB1	37.040 (37.4849)^{d}	40.370 (39.4444)^{cd}
CaB2	32.590 (34.8070)^{e}	42.960 (40.9503)^{c}
CaB3	43.703 (41.3802)^{bc}	37.780 (37.9230)^{d}
CaB4	37.040 (37.4849)^{d}	31.113 (33.8989)^{e}
CaB5	53.333 (46.9130)^{a}	58.517 (49.9093)^{a}
CaB6	44.813 (42.0208)^{b}	52.220 (46.2734)^{b}
CaB7	40.370 (39.4444)^{cd}	30.370 (33.4373)^{e}
CaB8	30.370 (33.4373)^{e}	24.443 (29.6256)^{f}
Control	- (0.5730)^{g}	- (0.5730)^{g}

Values are mean of three replications. Data in parenthesis are arcsine transformed values. Data followed by the same letter in a column are not significantly different according to Duncan's multiple range test at p = 0.05.

Table 2. Effect of *Bacillus* isolates on chickpea seedling growth.

Isolate	Vigour index	
	Roll towel	Pot culture
CaB1	2890.00^{b}	1908.93^{bc}
CaB2	2895.00^{b}	2046.14^{b}
CaB3	1696.00^{d}	2000.00b
CaB4	2340.00^{c}	1786.16^{cd}
CaB5	3230.00^{a}	2412.84^{a}
CaB6	2329.20^{c}	2267.71^{a}
CaB7	2550.00^{c}	2044.72^{b}
CaB8	2349.00^{c}	2052.60^{b}
Control	1641.50^{d}	1622.22^{d}

Values are mean of three replications; Control: Seeds treated with water instead of bacteria; Data followed by the same letter in a column are not significantly different according to Duncan's multiple range test at p = 0.05.

R. bataticola (Table 1) which was significantly higher as compared to other isolates. The results are in concordance with the findings of Zaim et al. (2013) who has reported *in vitro* mycelial inhibition of *F. oxysporum* f.sp. *ciceri* by antagonistic *Bacillus* spp.

Apart from mycelial growth inhibition, plant growth promotion is an important trait of soil microorganisms for improving crop productivity. The eight selected isolates were tested for growth promotion of chickpea by standard roll towel method and by pot culture studies. The results of the study revealed that all the eight isolates increased the vigour index of chickpea seedlings as compared to the control (Table 2). The plant growth promoting activity of *Bacillus* has been previously reported by Rajendran et al. (2007).

Detection of antibiotic genes of *Bacillus* isolates

Production of antimicrobial compounds serves as a determinant to decide the ability of an organism to control plant diseases. The beneficial rhizobacteria *B. subtilis* is one of the best biocontrol agents because it produces lipopeptides *viz;* fengycin, iturin and surfactin which displayed multifaceted biocontrol activity against plant pathogens (Ongena and Jacques, 2008). These anti-microbial cyclic lipopeptides (LPs) *viz*; surfactin, iturin and fengycin are specifically interesting because of their high surface activities and antagonistic potential (Kim et al., 2004).

Specific PCR primers were employed for the detection of biosynthetic genes of multimodular enzymes, the peptide synthetases, involved in the synthesis of antifungal lipopeptides. The PCR amplified products, after separation in agarose gel electrophoresis followed by gel documentation, revealed the presence of surfactin, iturin, fengycin and bacillomycin D genes in the *Bacillus* strains. The surfactin gene was amplified at 440 bp, iturin gene at 648 bp, fengycin at 986 bp and bacillomycin D gene at 875 bp. Generally, many members of the *Bacillus* spp. are known producers of lipopeptides belonging to the surfactin, iturin and fengycin families.

The genes identified in each isolate in the present study are furnished in Table 3. Four of the eight isolates were found to produce all the four antibiotic genes. The isolate CaB5 which had all the four antibiotic genes and which showed maximum *in vitro* inhibition was selected for further studies. The 16s rRNA of the isolate CaB5 was sequenced and identified as *Bacillus subtilis* and the gene sequence is submitted at NCBI with the Accession No: KP412481.

Extraction of crude antibiotics and TLC

The production of lipopeptides by the *Bacillus* strain CaB5 was confirmed by extraction of crude antibiotics and tested by TLC. The results of TLC indicated the presence of two bands by UV visualization with one at R*f* value 0.4 and another at R*f* value 0.7. On spraying with 0.2% ninhydrin, bands were visible at 0.08, 0.67, 0.72

Table 3. List of antibiotic genes identified in each *Bacillus* isolate.

Isolates	Surfactin	Iturin A	Fengycin D	Bacillomycin D
CaB1	+	-	-	+
CaB2	+	+	+	+
CaB3	+	+	+	+
CaB4	+	+	-	+
CaB5	+	+	+	+
CaB6	+	+	+	+
CaB7	-	+	+	+
CaB8	-	+	+	-

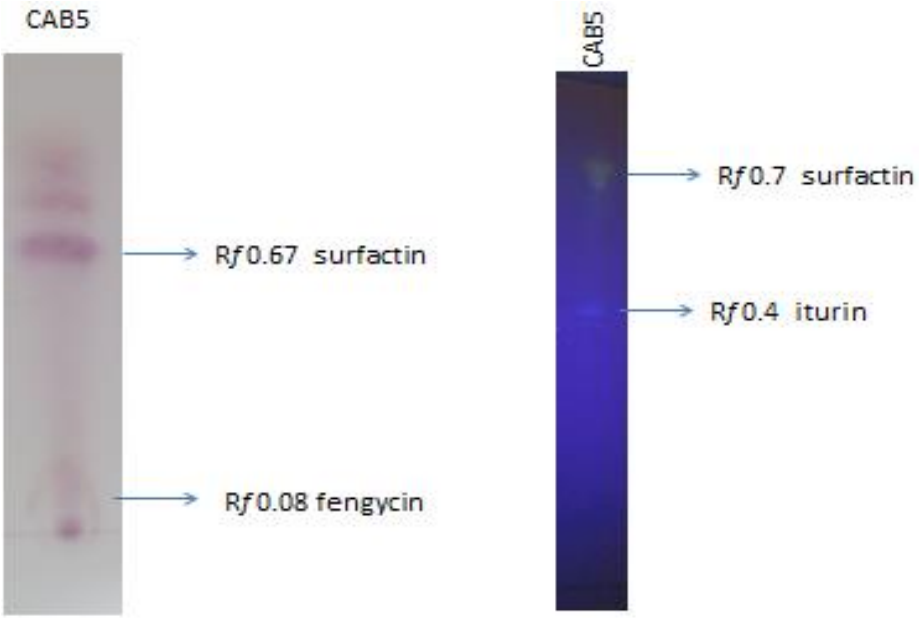

Figure 1. Thin layer chromatography of crude antibiotics of the *Bacillus* strain CaB5.

and 0.75 R*f* values (Figure 1). The crude antibiotics of *Bacillus amyloliquefaciens* PPCB004 developed spots with R*f* between 0.08 and 0.2 and were identified as fengycin, one spot at R*f* 0.6 was identified as iturin A, and the highest spot with R*f* 0.75 was identified as surfactin (Arrebola et al., 2010). Romero et al. (2007) has reported that antifungal effect of cell-free supernatants as well as the presence of the antifungal compounds bacillomycin, fengycin, iturin A, and surfactin are the key factors in antagonism of *B. subtilis* towards *Podosphaera fusca* causing powdery mildew of cucurbits.

In vitro assay of crude antibiotics

The effect of crude antibiotics on the root rot and wilt pathogens of chickpea was tested by agar well diffusion method. The crude antibiotics, at 50 µl volume inhibited the mycelial growth of *F. o*. f. sp. *ciceri* by 41.6% and *R. bataticola* by 40% (Figure 2). The members of the iturin family exhibit strong antifungal and haemolytic activities (Maget Dana and Peypoux, 1994). Fengycin shows specific antifungal activity against filamentous fungi and inhibits phospholipase A2 activity (Nishikiori et al., 1986). When the induction of morphological changes was assayed using crude antibiotics, bulb formation in the mycelia of *F. o*. f. sp. c*iceri* was observed after 6 h of growth, indicating production of antifungal compounds (Figure 3). Although there was germination of conidia, the germ tube formed was abnormal. Light microscopic examination of germinating spores and hyphal tips revealed shrunken, granulated and vesicular cytoplasm as compared to the hyaline, healthy cytoplasm of control hyphae. This was in concurrence with the studies conducted by Tendulkar et al. (2007) on the effect of *Bacillus licheniformis* extracts on *Magnaporthe grisea*.

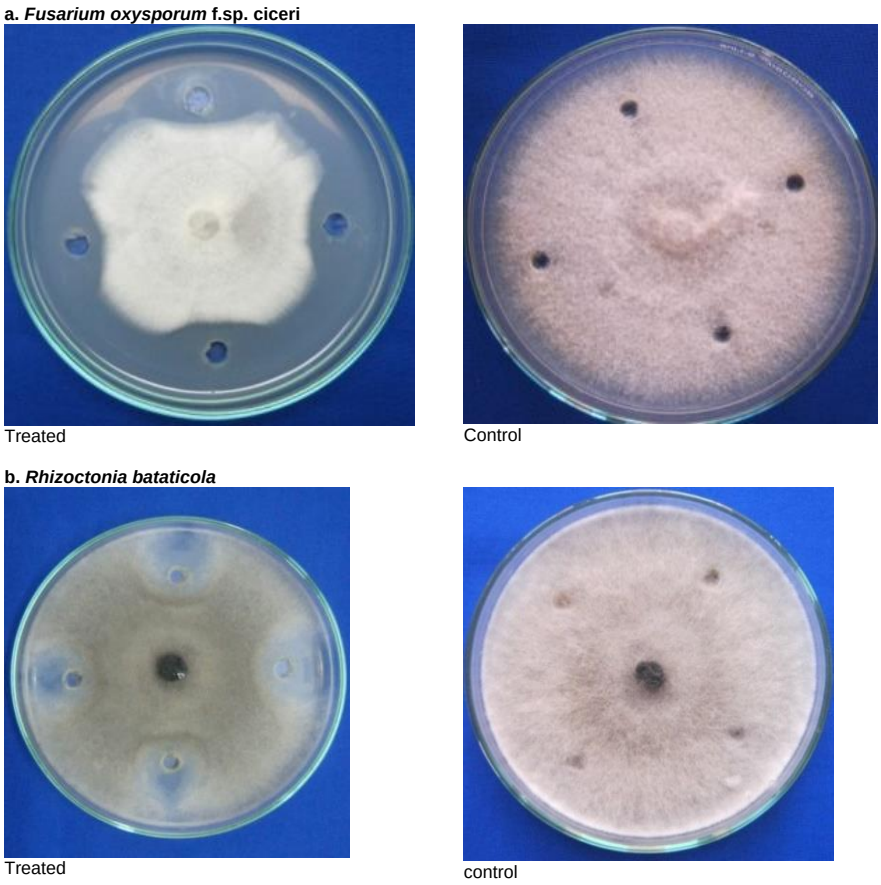

Figure 2. Agar well diffusion assay of crude antibiotics of the *Bacillus* strain CaB5.

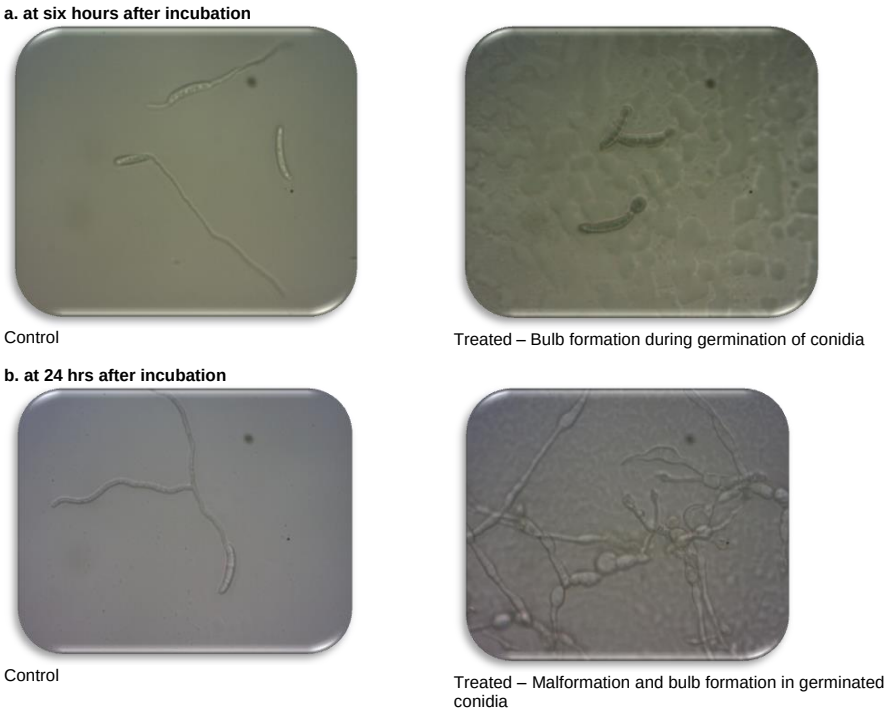

Figure 3. Spore germination inhibition assay of *F. oxysporum* f.sp. *ciceri* by cavity slide technique.

Conclusion

The use of beneficial microorganisms is considered as one of the most promising methods for more rational and safe crop management practices. Based on the results of the study, it is concluded that native *Bacillus* strains efficiently inhibited the growth of root rot (*R. bataticola)* and wilt (*F.o*. f.sp. *ciceri)* pathogens on chickpea by producing an array of lipopeptides like surfactin, iturin and fengycin and also enhanced the seedling vigour of chickpea. Hence *B. subtilis* strain CaB5 can be considered as a promising biocontrol agent for the management of root rot and wilt diseases of chickpea.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the funding from Department of Science and Technology, SERB, Government of India in carrying out this work.

REFERENCES

Arrebola E, Jacobs R, Korsten L (2010). Iturin A is the principal inhibitor in the biocontrol activity of *Bacillus amyloliquefaciens* PPCB004 against postharvest fungal pathogens. J. Appl. Microbiol.108:386-395

Athukorala SNP, Fernando WGD, Rashid KY (2009). Identification of antifungal antibiotics of *Bacillus* species isolated from different microhabitats using polymerase chain reaction and MALDI-TOF mass spectrometry. Can. J. Microbiol. 55:1021-1032

Baki AAA, Anderson JD (1973). Vigour determination in soybean seed by multiple criteria. Crop Sci. 31:630-633.

Dennis C, Webster J (1971). Antagonistic properties of species groups of *Trichoderma*: Production of non-volatile antibiotics. Trans. Brit. Mycol. Soc. 57:25-39.

Gomez KA, Gomez AA, (1984). Statistical Procedure for Agricultural Research. John Wiley and Sons, New York, p. 680

Gordillo MA, Maldonado MC (2012). Purification of peptides from *Bacillus* strains with biological activity. Chromatography and Its Applications. Dr. Sasikumar Dhanarasu (Ed.), pp. 201-225.

Islam MR, Jeong YT, Lee YC, Song CH (2012). Isolation and identification of antifungal compounds from *Bacillus subtilis* C9 inhibiting the growth of plant pathogenic fungi. Mycobiology 40(1):59-66.

ISTA (1993). Proceedings of the International Seed Testing Association, International Rules for Seed Testing. Seed Sci. Technol. 21: 25–30.

Johri BN, Sharma A, Virdi JS (2003). Rhizobacterial diversity in India and its influence on soil and plant health. Adv. Biochem. Eng. Biotechnol. 84:49-89.

Kim PI, Bai H, Chae H, Chung S, Kim Y, Park R, Chi YT (2004). Purification and characterization of a lipopeptide produced by *Bacillus thringiensis* CMB26. J. Appl. Microbiol. 97: 942-949.

Kloepper JW, Ryu CM, Zhang S (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology 94: 1259-1266.

Knapp JE, Chandlee JM (1996). RNA/DNA mini-prep from a single sample of orchid tissue. Biotechniques 21:54-56.

Maget-Dana R, Ptak M (1995). Interactions of surfactin with membrane models. Biophys. J. 68:1937-1943.

McKeen CD, Reilly CC, Pusey PL (1986). Production and partial characterization of antifungal substances antagonistic to *Monilinia fructicola* from *Bacillus subtilis*. Phytopathology 76:136-139.

Melody SC (1997). Plant Molecular Biology - A Laboratory Manual. Springer-Verlag, New York. pp. 121-154.

Nishikiori T, Naganwa H, Muraoka Y, Aoyagi T, Umezawa H (1986). Plipastatins: new inhibitors of phospholipase A2, produced by *Bacillus cereus* BMG302-fF67. III. Structural elucidation of plipasatins. J. Antibiot. 39(6):755-761

Ongena M, Jacques P (2008). Lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol. 16:115-125.

Pandey DK, Tripathi NN, Tripathi RO, Dixit SN (1982). Fungitoxic and phyototoxic properties of essential oil of *Phylis sauvolensis*. Pfkrankh Pfschuz. 89:334-46.

Rajendran L, Karthikeyan G, Raguchander T, Samiappan R (2007). *In vitro* evaluation of bacterial endophytes influence on *Ganoderma lucidum* (Leys) Karst. Mycelial growth. J. Plant Prot. Res. 47(4):425-436.

Ramarathnam R, Chen BSY, Fernando WGD, Xuewen G, de Kievit T (2007). Molecular and biochemical detection of fengycin- and bacillomycin D-producing *Bacillus* spp., antagonistic to fungal pathogens of canola and wheat. Can. J. Microbiol. 53(7):901-911.

Rangasamy R (1995). A Text Book of Agricultural Statistics. New Delhi, India: New Age International Publisher Ltd, 269 p.

Romero D, De Vicente A, Rakotoaly RH, Dufour SE, Veening JW, Arrebola E, Cazorla FM, Kuipers OP, Paquot M, Perez-Garcia A (2007). The iturin and fengycin families of lipopeptides are key factors in antagonism of toward *Podosphaera fusca*. Mol. Plant Microbe Interact. 20:430-440.

Saharan BS, Nehra V (2011). Plant growth promoting rhizobacteria: a critical review. Life Sci. Med. Res. p. 21.

Sambrook J, Fritsch EF, Maniatis T (1999). Molecular Cloning: A Laboratory Manual. 2nd ed. N.Y., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, 1659 p.

Stein T (2005). *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol. Microbiol. 56:845-857.

Tagg JR, McGiven AR (1971). Assay system for bacteriocins. Appl. Microbiol. 21:943.

Tendulkar SR, Saikumari YK, Patel V, Raghotama S, Munshi TK, Balaram P, Chattoo BBJ (2007). Isolation, purification and characterization of an antifungal molecule produced by *Bacillus licheniformis* BC98, and its effect on phytopathogen *Magnaporthe grisea*. Appl. Microbiol. 103(6):2331-2339.

Turner JT, Backman PA (1991). Factors relating to peanut yield increases after seed treatment with *Bacillus subtilis*. Plant Dis. 75:347-53

Vincent JM (1947) Distortion of fungal hyphae in presence of certain inhibitors. Nature 159:850.

Zaim S, Belabid L, Bellahcene M (2013). Biocontrol of chickpea fusarium wilt by *Bacillus* spp rhizobacteria. J. Plant Prot. Res. 53 (2):177-183.

academicJournals

Vol. 9(15), pp. 1105-1109, 15 April, 2015
DOI: 10.5897/AJMR2014.7302
Article Number: 693157452649
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Different dosages of SALMEX® to control *Clostridium perfringens* in poultry feed ingredients

Mariana Fröner Casagrande*, Marita Vedovelli Cardozo, Livia Boarini, Mariana Casteleti Beraldo-Massoli, Flávio Alves Longo, Juliano Vittori and Rubén Pablo Schocken-Iturrino

Veterinary Pathology Department, São Paulo State University – Campus of Jaboticabal, Rod. Prof. Paulo Donato Castelani s/n, Jaboticabal, SP, Brazil.

Received 27 November, 2014; Accepted 25 March, 2015

Ingredients of animal origin are important for the animal feed industry because they contain significant amounts of nutrients, minerals, and vitamins. However, the use of these raw materials is a problem due to pathogenic bacterial contamination, especially *Clostridium perfringens* and *Salmonella* spp. One way to control contamination is the addition of chemical products during ingredient production. Thus, the objective of this study was to evaluate a formaldehyde and organic acid-based product (SALMEX®) for two periods of action after experimental challenge with *C. perfringens* in two poultry feed ingredients. Microbiological analyses to enumerate the pathogen were conducted using colony-forming units per mL (CFU/mL) after incubation on SPS agar at 37°C for 48 h in anaerobic jars using the GasPak® system. The results show that there were significant differences among the dosage treatments and ingredients. With respect to the action time of the product, there were no significant differences observed between 24 h and 5 days, but there was a reduction in bacterial count with doses above 3 kg/t. This reduction was greater in the five-day SALMEX® treatment when compared to the 24-hour period. Thus, we can conclude that a higher product dose and a longer incubation time leads to more efficient product action.

Key words: Animal health, microbiology, nutrition, poultry industry, pathogen.

INTRODUCTION

Since the 1950s, the Brazilian poultry industry has undergone modernization, especially in the areas of genetics, animal management, nutrition, equipment and animal health, and has become highly productive (Tinôco, 2001). This sector is an important chicken meat exporter (Tavares and Ribeiro, 2007); according to ABEF 2014, Brazil is the world's third largest producer, behind only the USA and China, with 12.31 million tons produced, and the top exporter, with 3.918 million tons exported.

Ingredients originating from animals are made from meat byproducts that are not fit for human consumption, such as bone, feathers and blood. Because these byproducts are rich in nutrients, minerals and vitamins, they are important for the production of animal feed (Costa et al., 2008). However, these ingredients are also ideal environments for the proliferation of microorganisms, especially pathogens (Mazutti et al., 2008).

The main microorganisms present in such ingredients

*Corresponding author. E-mail: marianafcasagrande@yahoo.com.br. Tel: 55 16 3209 2652 / 55 16 3209 2600.

are *Salmonella* spp. and *Clostridium perfringens*, which are a problem because they can cause diseases in both animals and humans (Santos et al., 2008). Along with *Salmonella* spp. and *E. coli,* researchers have found *C. perfringens* in many types of feed ingredients, including meat meal, fish meal, corn, barley, wheat, and sunflowers (Prió et al., 2006).

C. perfringens is a Gram-positive, anaerobic bacterium that is capable of producing endospores (Schocken-Iturrino et al., 2009). It can be found throughout the environment and is frequently found in the intestines of domestic animals. It is responsible for various diseases, such as food poisoning and gas gangrene in humans and necrotic enteritis in poultry, which are caused by the toxins that this bacterium produces. Thus, adequate microbiological control in both the raw material used for feed and the final feedstuff product is important (Longo et al., 2010).

The primary method used to reduce animal feed contamination is to monitor and control bacterial contamination of ingredients and equipment used in the manufacturing and processing of the raw material (Wales et al., 2010). However, microbiological control can also be achieved with the addition of chemical products to the feed (Dibner and Buttin, 2002). Some chemicals that can control bacterial proliferation are organic acids (acetic acid, propionic acid, and salts of citric and formic acid), ethanol, formaldehyde, alcohol, zinc propionate, and zinc acetate, but the efficiency of these compounds can vary (Wales et al., 2010).

This study investigated the effect of different dosages (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 kg/t) of SALMEX®, a mixture of formaldehyde and organic acids, on the control of *C. perfringens* in two poultry feed ingredients (meat and bone meal; vegetable mix) for two periods of time (24 h and 5 days) after experimental challenge.

MATERIALS AND METHODS

Experimental tests with different dosages of chemical additive

This experiment used sixteen (16) samples of meat and bone meal and 16 samples of vegetable mix (40% soybean meal and 60% corn) at two different time-periods (24 h and five days), for a total of 64 samples. Four samples of each meal were used as positive controls for both 24 h and 5 days; these samples did not receive a bacteria inoculum, the chemical, nor the sterilization treatment. The remaining samples were placed in bags, sterilized at 121°C for 15 min in an autoclave and cooled by manual agitation to avoid compaction. After this process, the meal was inoculated with a bacterium and treated with chemical product, as detailed below.

The inoculum was obtained from a standard culture of *C. perfringens,* ATCC 13124. These cells were grown in Brain Heart Infusion (BHI) broth, incubated at 37°C for 48 h in anaerobic jars using the GasPak® System (BBL, USA), and centrifuged at 2,991 x *g* for 5 min to increase the cells concentration to 10^6 CFU/mL. The sediment was collected and the cells counted by serial dilution (to 10^{-6}) on Sulfite Polymyxin Sulfadiazine (SPS) agar and incubated under the same conditions as the BHI broth. The inoculum was maintained at 4ºC until the time of challenge (APHA, 2001).

For the SALMEX® treatments, each type of meal was divided into four units weighing 3 kg per unit for each dosage level. The SALMEX® (Btech, Brazil) contained 9% propionic acid, 33% formaldehyde and terpenes such as dispersants and surfactants. Next, 60 mL of *C. perfringens* inoculum was mixed with each meal portion to yield a final concentration of 10^4 CFU/mL.

The feed ingredients and SALMEX® were mixed in an experimental mixer that sprays the products while the ingredients are under rotation. This experimental machine was provided by the SALMEX® manufacturer and was specifically made for dosing and mixing fluids. Before each ingredient was added to the mixer, the equipment was cleaned and disinfected with 70% alcohol.

The dosages of SALMEX® were 1.0, 2.0 and 3.0 kg/t for the vegetable mix and 4.0, 5.0 and 6.0 kg/t for the animal meal. All samples were stored in the laboratory at room temperature for either 24 h or 5 days.

At the end of each period, 25 g of each sample was transferred to 225 mL of 1% peptone water, and serial dilutions were made to 10^{-6} for CFU enumeration. Each diluted sample was heat-shocked at 80°C for 10 min to allow the spores to germinate and to remove contaminants and then cooled in ice water. An aliquot of 1 mL of each dilution was transferred to a Petri dish, and SPS agar was added by the pour plate method. The plates were then incubated in anaerobic jars using the GasPak® System at 37°C for 48 h (APHA, 2001). Colonies suggestive of *C. perfringens* were transferred into test tubes containing BHI and subjected to the following biochemical tests: lactose, maltose and sucrose fermentation, salicin, indole, nitrate, gelatinase, motility and H_2S production (Carter et al., 1995).

Statistical analysis

The data from the count of colony forming units (CFU/mL) were statistically analyzed using an analysis of variance and means with a comparison by 8x2 factorial trial. The *F*-test was also performed, and the significance levels at 5% were determined. The statistical analysis was performed using AgroEstat, Version 1.0 (Barbosa and Maldonado Jr, 2010).

RESULTS AND DISCUSSION

Microorganisms, such as *Salmonella* spp. and *C. perfringens*, proliferate in ingredients of both animal and vegetable origin, and this contamination happens mostly in raw material (Cardozo et al., 2012; Casagrande et al., 2013). In the positive controls, those without chemical treatment *C. perfringens* growth were found. The average *C. perfringens* population for the vegetable mix was 4.28 log CFU/mL at 24 h and 4.64 log CFU/mL after 5 days. For the meat and bone meal control, the average was 4.46 and 4.45 log CFU/mL at 24 h and 5 days, respectively.

The three SALMEX® dosages for the vegetable mix were 1.0, 2.0 and 3.0 kg/t. After 24 h of chemical action, the population means were 3.27, 3.59, and 4.04 log CFU/mL, respectively. After five days of the product's action, the average population was 4.11 log CFU/mL for the dose of 1.0 kg/t, 3.43 log CFU/mL for 2.0 kg/t, and 2.57 log CFU/mL for 3.0 kg/t, as shown in Table 1.

For the meat and bone meal, the mean bacterial counts 24 h after the application of the chemical were 2.10 log CFU/mL for the dose of 4.0 kg/t, 3.48 log CFU/mL for 5.0 kg/t, and 4.14 log CFU/mL for 6.0 kg/t. On the fifth day of

Table 1. The levels of *Clostridium perfringens* in experimentally inoculated vegetable meal, and meat and bone meal, for periods of 24 hours and 5 days after the action of the SALMEX® product at predetermined dosages, presented in log CFU/mL.

Type of meal	Dosage of SALMEX® (Kg/t)	24 h	5 days	Type of meal	Dosage of SALMEX® (Kg/t)	24 h	5 days
VM[1]	0.0	4.18	4.51	MBM[2]	0.0	4.65	4.08
VM	0.0	4.08	4.32	MBM	0.0	4.51	4.60
VM	0.0	4.48	5.70	MBM	0.0	3.95	4.41
VM	0.0	4.30	6.08	MBM	0.0	4.48	4.54
Mean	-	4.28	4.64	Mean	-	4.46	4.45
SEM	-	0.07	0.38	SEM	-	0.13	0.10
VM	1.0	3.00	0.00	MBM	4.0	0.00	0.00
VM	1.0	3.64	0.00	MBM	4.0	0.00	0.00
VM	1.0	2.48	4.51	MBM	4.0	0.00	0.00
VM	1.0	3.23	4.32	MBM	4.0	2.70	0.00
Mean	-	3.27	4.11	Mean	-	2.10	0.00
SEM	-	0.21	1.10	SEM	-	0.58	0.00
VM	2.0	3.43	2.60	MBM	5.0	0.00	0.00
VM	2.0	0.00	3.88	MBM	5.0	0.00	0.00
VM	2.0	4.08	0.00	MBM	5.0	3.60	3.00
VM	2.0	2.88	3.48	MBM	5.0	3.90	3.30
Mean	-	3.59	3.43	Mean	-	3.48	2.88
SEM	-	0.78	0.76	SEM	-	0.94	0.79
VM	3.0	0.00	3.18	MBM	6.0	3.93	3.40
VM	3.0	4.49	0.00	MBM	6.0	4.34	0.00
VM	3.0	4.04	0.00	MBM	6.0	4.36	0.00
VM	3.0	3.54	0.00	MBM	6.0	3.34	0.00
Mean	-	4.04	2.57	Mean	-	4.15	2.80
SEM	-	0.89	0.69	SEM	-	0.21	0.74

[1]VM = vegetable mix; [2]MBM = meat and bone meal.

SALMEX® action, the dosage of 4.0 kg/t inhibited the growth of *C. perfringens*, while the doses of 5.0 and 6.0 kg/t only resulted in lower growth, with respective values of 2.88 log and 2.80 log CFU/mL (Table 1). This unexpected result may have occurred because of ingredient compaction and poor homogenization in the manual application of the *C. perfringens* culture used for challenge. Some compaction of the ingredients may have occurred due to the heat and humidity produced during the autoclave sterilization. This could explain the agent's survival ability, as the chemical product cannot penetrate this compaction.

C. perfringens has the capacity to form spores (Schocken-Iturrino et al., 2009) which supports its resistance to chemicals. Therefore, a longer chemical exposure is required for the product to act on the bacterial cell. This can explain the fact that the average bacterial counts increased for the 24-h period. In addition, Ricke (2003) found that the type of microorganism, the change in superficial tension, and spore formation, all determine bacterial sensitivity to organic acid antimicrobial agents.

Animal meals provide a better environment for pathogenic microorganism development and present a higher risk of contamination (Longo et al., 2010; Mazutti et al., 2008) when compared to vegetable meals. Therefore, the samples of animal origin were treated with higher dosages of SALMEX® (4.0, 5.0 and 6.0 kg/t) than those of vegetable origin.

When used for the control of *Salmonella* spp., the SALMEX® product was effective and prevented the growth of this microorganism at dosages ranging from 1.0 to 6.0 l/ton (Albuquerque et al., 1998). In another study on Enterobacteriaceae control in swine feed, treatments using a mixture of propionic acid and formaldehyde were performed. That study analyzed three different concentrations (0.0, 1.0, 2.0, and 3.0 g/kg) and two periods (24 h and 14 days), and enterobacteria reduction was observed in the concentration of 3 g/kg at 14 days (Sbardella et al., 2014). However, in this experiment, we only found lower population counts of *C. perfringens* with SALMEX® dosages over 3.0 kg/t. Furthermore, complete control was observed only with the SALMEX® dose of 4.0 kg/t and then, only in the bone and meat meal, not the vegetable meal.

Table 2. The comparison between the statistical means of the positive control and the products receiving different dosages, in log CFU/mL.

Treatment[(1)]	Mean
1	2.824[abc]
2	2.722[bc]
3	2.259[c]
4	0.949[c]
5	2.201[c]
6	2.688[bc]
7	4.705[a]
8	4.404[ab]
F-test	7.91 (p<0.0001)
MSD [(2)] (5%)	1.931
Period	Mean
24 h	3.144[a]
5 days	2.544[a]
F-test	3.88 (p=0.0548)
MSD (5%)	0.613
Value of the *F*-test for the interaction	
Treatment *vs* Period	1.71 (p=0.1285)

[a, b, c] Means within a column with unlike superscripts differ significantly ($P < 0.05$). [(1)]1= Vegetable Mix with 1.0 Kg/t SALMEX®; 2= Vegetable Mix with 2.0Kg/t SALMEX®; 3= Vegetable Mix with 3.0 Kg/t SALMEX®; 4= Meat and Bone Meal with 4.0 Kg/t SALMEX®; 5= Meat and Bone Meal with 5.0 Kg/t SALMEX®; 6= Meat and Bone Meal with 6.0 Kg/t SALMEX®; 7= Positive Control Vegetable Mix; 8= Positive Control Meat and Bone Meal. [(2)]MSD= Minimum Significant Difference for means comparison.

Our *C. perfringens* colony counts in the SALMEX® treated samples demonstrated that the product was most effective at higher doses and with longer periods of action. This is similar to two studies found in literature. The first one by Cardozo et al. (2012) determined that SALMEX® at a dosage of 6 kg/t was effective in inhibiting *C. perfringens* in animal meal. The second one by Casagrande et al. (2013) evaluated the efficiency of different products containing formaldehyde and organic acids in the elimination of the same pathogen at concentrations of 3.0 and 6.0 kg/t in animal and vegetable ingredients of poultry feed.

Bacteria inhibition by organic acids occurs through the inside of cells and the dissociation of cations and anions. Cations are responsible for reducing the bacteria's internal pH, consuming vital energy, and causing the death of these cells. The anionic form diffuses freely through the cell wall, and becomes toxic in this dissociated form (Lambert and Stratford, 1999). Finally, the antimicrobial action of organic acids is specifically related to the acid concentration, the pH of the environment and the type of microorganism (Wales et al., 2010; Dibner and Buttin, 2002).

Formaldehyde is a potent chemical product because of the way it operates in the cells. According to Tortora et al. (2005), formaldehyde has the ability to inactivate cellular constituents such as protein and nucleic acid. Thus, this product results in the death of the cell and is more effective in eliminating bacteria.

Statistical analysis of this experiment showed that, among the evaluated variation factors, the only significant difference was found between the treatments with p<0.0001. The product action period did not further affect the final result (p=0.0548). The interaction between treatment and time was not significant (p=0.1285), demonstrating that these factors are independent of each other. The statistical ANOVA showed a mean of 2.84 log CFU/mL, an SD of 1.22 and a CV of 42.865.

The statistical average for the different time-period treatments (24 h and 5 days) demonstrated that treatment of the vegetable mix with the dosage of 2.0 kg/t and the meat and bone meal with the dosage of 6.0 kg/t were statistically equivalent. The vegetable mix (3.0 kg/t) and the meat and bone meal (4.0 and 5.0 kg/t) treatments all obtained satisfactory and similar results (Table 2).

The positive controls for both the vegetable mix and the animal meal had higher average scores than the other treatments and were statistically equal. Interestingly, the treatment of the vegetable mix with the dosage of 1.0 kg/t showed results similar to all others. For the period of product action there were no significant differences observed between the 24 h and 5-day periods. In a study by Carrique-Mas et al. (2007), in which the periods of product action were 24 and 72 h, the effect of time also interfered with the effectiveness of treatments.

A study by Albuquerque et al. (1998), comparing different commercial organic acid compounds at different doses during experimental inoculation of *Salmonella* spp. in animal feed, concluded that organic acids show different bactericidal behaviors because their effectiveness depends on the product and the concentration used.

Conclusion

SALMEX® reduced *C. perfringens* populations in samples that received doses above 3.0 kg/t. After 24 hours of product action, there were no counts in 29% of the samples, and after a period of five days, this percentage increased to 63%. This demonstrates that time is an important factor for SALMEX® action.

Conflict of Interests

The authors did not declare any conflict of interests.

ACKNOWLEDGMENTS

The authors appreciate the help of Dr. José Carlos Barbosa, Professor of Statistics, São Paulo State University (UNESP), Jaboticabal Campus, SP, Brazil. We

are grateful to the Btech Company for their donation of the chemical additives, to Coordination for the Improvement of Higher Education Personnel, Brazil (CAPES) and to São Paulo Research Foundation, Brazil (FAPESP) for the financial support.

REFERENCES

ABEF - Associação Brasileira dos Produtores e Exportadores de Frango. Relatório Anual 2014. WebMD. Acessed Fev. 2015 <http://www.ubabef.com.br/publicacoes>

Albuquerque R, Ito NMK, Miyaji CI (1998). Tratamento de rações de aves com ácidos orgânicos: estudo da atividade bacteriana e avaliação de técnicas de recuperação de *Salmonella* spp. J. Vet. Res. An. Sci. 35(6):279-282.

APHA, American Public Health Association (2001). Compendium of methods for the microbiological of foods. 4th ed. Washington.

Barbosa JC, Maldonado Jr W (2010). AgroEstat – Sistema para Análises Estatísticas de Ensaios Agronômicos, Versão 1.0.

Cardozo MV, Schocken-Iturrino RP, Beraldo-Massoli MC, Cavani R, Casagrande MF, Boarini L, Borges CA, Beraldo LG (2012). Pathogens in animal meal and the use of Salmex® in the elimination of *Clostridium perfringens*. Afr. J. Microbiol. Res. 6(16):3727-3731. http://dx.doi.org/10.5897/AJMR12.185

Carrique-Mas JJ, Bedford S, Davies RH (2007). Organic acid and formaldehyde treatment of animal feeds to control *Salmonella*: efficacy and masking during culture. J. Appl. Microbiol. 103: 88-96. http://dx.doi.org/10.1111/j.1365-2672.2006.03233.x

Carter GR, Chengappa MM, Roberts AW (1995). Essentials of Veterinary Microbiology, 5th ed., Williams and Wilkins, Baltimore. P. 394.

Casagrande MF, Cardozo MV, Beraldo-Massoli MC, Boarini L, Longo FA, Paulilo AC, Schocken-Iturrino RP (2013). *Clostridium perfringens* in ingredients of poultry feed and control of contamination by chemical treatments. J. Appl. Poult. Res. 22(4):771-777. http://dx.doi.org/10.3382/japr.2012-00707

Costa DPS, Romanelli PF, Trabuco E (2008). Aproveitamento de vísceras não comestíveis de aves para elaboração de farinha de carne. Ciênc. Tecn. Alim. 28(3):746-752.

Dibner JJ, Buttin P (2002). Use of organic acids as a model to study the impact of gut microflora on nutrition and metabolism. J. Appl. Poult. Res. 11(4):453-463. http://dx.doi.org/10.1093/japr/11.4.453.

Lambert RJ, Stratford M (1999). Weak acid preservatives: modeling microbial inhibition and response. J. Appl. Microbiol. 86:157-164. PMID:10030018.

Longo FA, Silva IF, Lanzarini MA (2010). A importância do controle microbiológico em rações para aves. In: Simpósio Brasil Sul de Avicultura, Chapecó, SC. Anais, pp. 1-23.

Mazutti MA, Treichel H, Di Luccio M (2008). Esterilização de farinha de subprodutos animais em esterilizador industrial. Ciênc. Tecnol. Aliment. 30(1):48-54.

Prió P, Gasol R, Soriano RC, Perez-Rigau A (2006). Effect of raw material microbial contamination over microbiological profile of ground and pelleted feeds. In: Brufan J. (Ed.): From Feed to Food, pp. 197-199.

Ricke SC (2003). Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. Poult. Sci. 82:632-639. PMID: 12710485.

Santos JRG, Conceição FR, Gil-Turnes C (2008). Enterite necrótica aviária. Ciênc. Rur. 38(7):2076-2082. http://dx.doi.org/10.1590/S0103-84782008000700047.

Sbardella M; Perina DP; Andrade C; Longo FA; Miyada VS (2014) Effects of a dietary added formaldehyde-propionic acid blend on feed enterobacteria counts and on growing pig performance and fecal formaldehyde excretion. Ciência Rural, Santa Maria, Online version. Acessed in Fev. 2015. http://dx.doi.org/10.1590/0103-8478cr20131660

Schocken-Iturrino RP, Ishi M, Vittori J (2009). Clostridioses em aves. 533-550, In: Berchieri AJR, Silva EN, Di Fábio J, Sesti L, Zuanaze MAF. Doenças das aves. Campinas: Facta, 2 ed., Campinas - SP.

Tavares LP, Ribeiro KCS (2007). Desenvolvimento da avicultura de corte brasileira e perspectivas frente à influenza aviária. Org. Rur. Agroind. 9(1):79-88.

Tinôco IFF (2001). Avicultura Industrial: Novos Conceitos de Materiais, Concepções e Técnicas Construtivas Disponíveis para Galpões Avícolas Brasileiros. Rev. Bras. Ciênc. Avíc. 3(1):01-26. http://dx.doi.org/10.1590/S1516-635X2001000100001.

Tortora GJ, Funke BR, Case CL (2005). Microbiology. 920p. 8ª ed. ARTMED. Porto Alegre, Brasil.

Wales AD, Allen VM, Davies RH (2010). Chemical treatment of animal feed and water for the control of *Salmonella*. Food. Path. Dis. 7(1):3-15. http://dx.doi.org/10.1089/fpd.2009.0373 PMID: 19821738.

academicJournals

Vol. 9(15), pp. 1110-1117, 15 April, 2015
DOI: 10.5897/AJMR2014.7357
Article Number: 7ED6A7752651
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Bacterial inoculation effect on soil biological properties, growth, grain yield, total phenolic and flavonoids contents of common buckwheat (*Fagopyrum esculentum* Moench) under hilly ecosystems of North-East India

Raghavendra Singh[1]*, Subhash Babu[1], R. K. Avasthe[1], G. S. Yadav[2], Tirtha Kumari Chettri[1], C. D. Phempunadi[1] and Tarama Chatterjee[3]

[1]ICAR Research Complex for NEH Region, Sikkim Centre, Tadong, Gangtok, Sikkim, India.
[2]ICAR Research Complex for NEH Region, Tripura Centre, West Tripura, India.
[3]Regional Research Station, Hill Zone, UBKV, Kalimpong, West Bengal, India.

Received 31 December, 2014; Accepted 25 March, 2015

Field experiments were carried out at Research Farm, ICAR Sikkim Centre, Tadong during two consecutive *Rabi* seasons of 2012 and 2013 to determine the effect of different microbial inoculants on selected soil biological properties, growth, yield, and quality of common buckwheat, and then identify the best inoculant for application for local common buckwheat production in hilly ecosystem of North-East India. The results indicated that seed inoculants applied to common buckwheat effectively increased plant growth, chlorophyll content (SPAD), yield attributing characters, total phenolic and flavonoid content, grain yield, and soil biological properties. Among the different inoculations, combined application of *Azotobacter* spp. and *Azospirillum* spp. was found most efficient and resulted in maximum values of plant growth parameter, yield attributing characteristics, grain yield (1.23 Mg/ha), soil microbial biomass carbon (SMBC) and dehydrogenase activities at all the growth stages of common buckwheat.

Key words: Buckwheat, dehydrogenase activities, flavonoid content, phenolic content, microorganisms, yield.

INTRODUCTION

Buckwheat (*Fagopyrum* spp.) is a unique traditional food crop of tribes of Himalayan region of North East India. It occupies about 90% of cultivated lands in the higher Himalayas with a solid stand. It is a short duration crop (2-3 months) and fits well in the high Himalayan ecosystems where a crop's growing season is limited period because

*Corresponding author Email: raghavenupc@gmail.com.

of early winter and heavy snow fall. In the higher Himalayas, up to 4500 m, buckwheat is the only crop grown (Joshi and Paroda, 1991). Buckwheat seems able to use insoluble phosphorus and potassium in soil and produces good seed yields, even on less fertile soils (Kontturi et al., 2004). Buckwheat possesses tolerance ability against drought, poor soil and extreme environments and has wide potential for adapting to climate change (CGIAR, 2013). Among the buckwheat genotypes cultivated in North East India, common buckwheat locally known as *Meethey Phapar* (*Fagopyrum esculentum*) is gaining more popularity due its taste and shorter growth period. North East region of India is designated as natural economic zone and opportunity zone for organic farming. Buckwheat concerns to dietary food crops with high nutritional value in respect of protein content (Krzysztof et al., 2012) with optimum combination of irreplaceable amino acids, vitamins, macro- and micro-elements, and enzymes. Buckwheat is a unique crop, which contains vitamin P (Pirogovskaya et al., 2004). Recently, its cultivation area has gradually decreased, largely because of low yield and profit to farmers. There are several reasons for low productivity of buckwheat in the region, among them proper nutrition to the crop is most the important one. There is ample scope for increasing production of buckwheat with the use of good agronomic practices as well as proper fertility management. Hence, there is an urgent need to conduct research to allow for an increase in buckwheat production in the region. Most of the studies on buckwheat have focused on breeding and cultivation; research on buckwheat fertilization has mostly concentrated on chemical fertilizers and their effect (Zhang et al., 2001). However, fertilizer application sometimes causes crop lodging in results in yield reduction. Some research has shown that the application of microorganisms could increase soil nutrient supply and stimulate plant growth (Tao et al., 2004). There is very limited or no information available on the effect of micro-organism inoculation on growth, yields, quality and soil biological properties of common buckwheat under hilly ecosystems. Therefore, the present investigation was carried out to find out the best inoculant of common buckwheat for enhancing the productivity under the Himalayan region of North East India.

MATERIALS AND METHODS

Details of experimental field

Field experiments were carried out during two consecutive *Rabi* (Winter) seasons of 2012 and 2013 at experimental block of Research Farm, ICAR Research Complex for NEH Region Sikkim Centre, Tadong, situated at a latitude of 27°32' N and longitude of 88°60' E, altitude of 1300 m above mean sea level (amsl). The average rainfall received during the period of investigation was 143.5 mm and the region hardly receives any rainfall during cropping period (winter season). Soils of experimental field were clay loam and belongs to Inceptisol and had soil pH 5.7 (1: 2.5 soil and water ratio), 226.3 kg/ha alkaline permanganate oxidizable N, 23.40 kg/ha Brays P_1, 199.7 kg 1 *N* ammonium acetate exchangeable K and 1.93% organic carbon.

Experimental design and treatments

In order to evaluate the effects of selected inoculants on the growth, productivity, quality and soil biological properties of common buckwheat, 100 g of buckwheat seed for each plot was treated one day before sowing with 25 ml of different inoculants or a mixture as designed ($3x10^9$ cfu/ml in case of microorganism). In plots of 3.0 m x 4.0 m, the seeds were sown in four replications at spacing of 30 cm between rows, 10 rows per plot in a completely randomized block design. Seed sown was 25 g/plot. The experiment comprises six treatments *viz.,* control, cow urine, *Azospirillum* spp., *Azotobacter* spp., *Azotobacter* spp. + *Azospirillum* spp. and *Azotobacter* spp. + *Azospirillum* spp. + cow urine. Sowing was done in shallow furrows made with the help of wooden plough (*Desi* country plough made of woods and a shovel) /and the seeds were sown in line, prior to sowing, 1.8 kg/plot of vermicompost were applied irrespective of treatments, and no other manure were applied during the experiment. Thinning was done at 15 days after sowing (DAS) to maintained optimum plant population. The crop was sown on 5th and 8thNovember in 2012 and 2013, respectively as per the recommended practices and harvested on 24th and 28th February during 2013 and 2014, respectively. Observation on growth viz. plant height (cm), stem girth (cm), leaves/plant plant, root length (cm), root dry weight (g/plant) and top dry weight (g/plant) and yield parameters were recorded as per the standard procedure. Similarly, chlorophyll content in leaves of buckwheat was determined by using SPAD (CCM-200) at 30, 60 and 90 DAS in morning hour, during both years.

Quality analysis

Preparation of extracts

Buckwheat flour (2 g) from raw samples was homogenized with 20 ml of 80% ethanol. The mixture was kept in agitation for 30 min at 160 rpm in an orbital shaker. Then, the homogenate was centrifuged for 10 min at 11000 rpm and the supernatant was removed, filtered (0.45 µm) and stored at -18°C for analysis.

Estimation of total phenolic content (TPC)

TPC in extracts was determined using Folin-Ciocalteau reagent, following the method described by Singleton et al. (1999). The liquid extracts were diluted and mixed with Folin-Ciocalteau reagent (2 ml) and 20% sodium carbonate solution. The mixture was incubated in the dark for 1 h at room temperature (25ºC). After incubation, absorbance was measured at 525 nm using spectrophotometer. The results were expressed as mg equivalent of Gallic acid (GAE) per 100 g of dry matter (QE).

Estimation of total flavonoid content (TFC)

TFC were measured by method of Zhishen et al. (1999) using Quercetin standard. Briefly, 0.5 mL of aliquot of extract was added to 75 µL of 5% $NaNO_2$ solution. After 6 min, 150 µL of 10% $AlCl_36H_2O$ solution was added and the mixture was allowed to stand another 5 min. Then, 0.5 mL of 1 mol/l NaOH and 2.5 mL of distilled water was added. The solutions were mixed and absorbance was measured at 510 nm using spectrophotometer. Total flavonoid content of extracts was expressed as mg of quercetin/100 g of dry matter (QE).

Estimation of soil biological properties

Soil sample were taken from crop root (0-15 cm soil depth) by core sampler at 30, 60 DAS and at harvest of buckwheat. The soil samples were air dried and kept in freezer (-20ºC) until the analysis of soil biological properties. Estimation of soil biological properties such as dehydrogenase activity and soil microbial biomass carbon were done per the procedures describe below.

Dehydrogenase activity

Dehydrogenase activity of soil samples was estimated by the method described by Casida et al. (1964).

Reagents

Triphenyl-tetrazolium chloride (TTC): TTC (3.0 g) was dissolved in 100 ml distilled water and stored in an amber coloured bottle at 4°C; methanol (AR grade); Standard triphenyl formazan (100 μg/ml): 10 mg triphenyl (TPF) dissolved in 100 ml distilled water.

Procedure of estimation

Fresh air-dried soil sample (6 g) was saturated with 1.0 ml freshly prepared TTC (3% w/v) solution in a screw capped test tube to which pinch (0.1 g) of $CaCO_3$, was added. Care was taken that no air bubble remained during packing of soil sample and rotated gently by shaking. These test tubes were incubated at 28±1°C (28-30°C) for 24 h. After 24 h, TPF was extracted (pink layer). 10 ml Methanol was added to these test tubes and rotated it well for 1 min /sample. The supernatant was taken out carefully after allowing standing for 10 minutes. Absorbance of supernatant was recorded by Spectrophotometer at 485 nm. A standard curve was prepared with TPF (0-50 μg/ml). Concentration of TPF in sample was calculated with standard curve. Dehydrogenase activity was calculated and expressed in terms of μg TPF liberated g/soil/h or μg TPF g/soil/day.

$$\text{Dehydrogenase activity } (\mu \text{ TPF g/soil/day}) = \frac{\text{Concentration reading of spectrophotometer}}{6}$$

Microbial biomass carbon (MBC)

Microbial biomass carbon in soil samples was estimated by the method described by Vance et al. (1987) and Numan et al. (1998) derived a method for estimation of microbial biomass C.

Reagent

Chloroform; 0.5 M K_2SO_4: Prepared by adding 87.135 g of K_2SO_4 in 1 L distilled water.

Procedure of estimation

Soil sample (17.5 g) was taken in a closed-capped bottle and 1.0 ml of chloroform was added and fumigated these samples and one non fumigated set was also prepared in a 250 ml flask. After that, these incubated samples were kept in dark for 24 h. After 24 h of incubation, chloroform was evaporated at 50°C in BOD that is the caps were opened for next 20-24 h. After that 70 ml 0.5 M K_2SO_4 was added to samples and shaken for 30 min. Supernatant was taken out by filtering the samples with Whatman No. 42 filter paper. Absorbance of supernatant was recorded immediately for both fumigated and non-fumigated at 280 nM. Soil microbial biomass carbon (SMBC) was calculated and expressed as mg kg/soil.

Statistical analysis

All the data obtained was statistically analysed using the *F*-following Gomez and Gomez (1984). CD values at $P = 0.05$ were used to determine the significance of difference between treatment means.

RESULTS

Effect of inoculation on growth of common buckwheat

Mean data of two years showed that in general, plant height (Table 1a), root length, root dry and top dry weight accumulation (Table 1b) increased with the age of crop and achieved to the maximum at maturity except leaves/plant and stem girth, which recorded increase only up to 90 DAS. Initially, plant growth in terms of plant height, stem girth, leaves/plant, root length, root and top dry weight accumulation was slow up to 30 DAS. Thereafter, the rate of increase reached a peak between 30 and 60 days and declined towards maturity. Inoculation has significant effect on all growth parameters of common buckwheat under study. Among the inoculations, combined application of *Azospirillum* spp. and *Azotobacter* spp. resulted in significant higher values of plant height (cm), stem girth (cm), leaves/plant, root length (cm), root dry and top dry weight accumulation (g/plant) at all the growth stages of crop. However, it remained statistically at par with single application of *Azospirillum* spp. at 90 DAS in terms of plant height, at 30 DAS in terms of stem girth and leaves/plant, at 30 DAS and 60 DAS in terms of root length root and aerial part dry weight accumulation during the course of study.

Effects of inoculation on chlorophyll content (SPAD), yield attributes and yields of common buckwheat

In general, irrespective of treatments, chlorophyll content in leaves of common buckwheat increased linearly from 30 to 60 DAS declined thereafter. Seed inoculation with different substrate showed the significant effect on chlorophyll content in common buckwheat at all the growth stages (Figure 1). Among the inoculations, combined application of *Azospirillum* spp. and *Azotobacter* spp. recorded significantly higher SPAD values at all the growth stages except at 60 DAS; at this stage, it remained statistically at par with the single application of *Azospirillum*. With respect to yield attributes and yield, seed inoculation showed significant effect over control. Among the treatments, combined inoculation of *Azospirillum* spp. and *Azotobacter* spp. resulted in

Table 1a. Inoculation effect on growth of common buckwheat (Mean data of 2 years).

Treatment	Plant height (cm)				Stem girth (cm)				Leaves/plant			
	30 DAS	60 DAS	90 DAS	At harvest	30 DAS	60 DAS	90 DAS	At harvest	30 DAS	60 DAS	90 DAS	At harvest
Control	15.37	55.8	73.4	109.7	0.21	0.56	0.63	0.62	2.75	5.25	12.25	11.10
Cow urine	14.97	61.6	72.7	116.2	0.22	0.57	0.65	0.64	4.50	6.50	14.75	13.50
Azospirillum	15.22	64.4	84.9	121.2	0.26	0.60	0.71	0.65	6.00	7.25	17.75	15.25
Azotobacter	16.90	62.6	86.7	121.0	0.25	0.59	0.70	0.66	4.75	6.75	19.00	16.50
Azospirillum+*Azotobacter*	17.72	66.9	90.0	126.2	0.28	0.63	0.75	0.71	6.25	8.25	21.75	20.00
Azospirillum+*Azotobacter*+Cow urine	16.40	62.7	84.0	116.5	0.23	0.57	0.68	0.62	4.00	5.50	18.25	15.00
SEM±	0.99	1.14	1.81	1.37	0.01	0.005	0.01	0.012	0.44	0.32	0.63	0.43
CD (*P*=0.05)	2.98	3.45	5.60	4.13	0.02	0.015	0.03	0.037	1.34	0.97	1.89	1.32

Table 1b. Inoculation effect on growth of common buckwheat (Mean data of 2 years).

Treatment	Root length (cm)				Root dry weight (g/plant)				Top dry weight (g/plant)			
	30 DAS	60 DAS	90 DAS	At harvest	30 DAS	60 DAS	90 DAS	At harvest	30 DAS	60 DAS	90 DAS	At harvest
Control	1.99	8.75	10.18	11.00	0.106	0.255	0.375	0.825	0.620	2.29	3.50	5.52
Cow urine	2.05	9.80	11.63	12.60	0.110	0.271	0.405	0.870	0.683	2.35	3.58	5.81
Azospirillum	2.94	10.80	12.40	13.00	0.120	0.284	0.423	0.945	0.866	2.41	3.70	6.15
Azotobacter	2.81	9.68	11.98	12.75	0.115	0.262	0.428	0.908	0.782	2.40	3.60	5.73
Azospirillum+*Azotobacter*	3.05	11.10	12.97	14.50	0.125	0.297	0.480	0.968	0.890	2.60	3.82	6.71
Azospirillum+*Azotobacter*+Cow urine	2.66	10.15	11.43	12.50	0.112	0.265	0.415	0.875	0.761	2.31	3.52	6.17
SEm±	0.03	0.12	0.18	0.45	0.002	0.004	0.008	0.009	0.010	0.06	0.02	0.13
CD (*P*=0.05)	0.10	0.36	0.54	1.34	0.005	0.013	0.024	0.027	0.030	0.17	0.07	0.40

maximum number of seeds/plant (135), seed yield/plant (2.99 g), test weight (23.65 g), and grain yield (1.23 Mg/ha) over other treatments (Table 2).

Effect of inoculation on total phenolic and flavonoid contents of buckwheat seed

Mean data of two years pertaining to total phenolic and flavonoids content is depicted in Figure 2. Seed inoculation had significant effect on total phenolic and flavonoids content in seed of common buckwheat. All the treatments significantly enhanced the total phenolic and flavonoids content in seed over control. Among the treatments, combined application of *Azospirillum* spp. and *Azotobacter* spp. recorded the highest in total phenolic (17.20 mg GAE/100 g) and flavonoid (5.28 mg QE/100 g) contents were in tune of 19.77 and 26.31% increment over the control (no inoculation).

Effect of inoculation on dehydrogenase activities and soil microbial biomass carbon

Mean data of two years presented in Figure 3 showes that soil microbial biomass carbon (SMBC) and dehydrogenase activity registered marked increase with the advancement in crop growth stages up to harvest. During the experiments it was found that among the treatments, significantly higher value of soil SMBC and dehydrogenase activity recorded with the

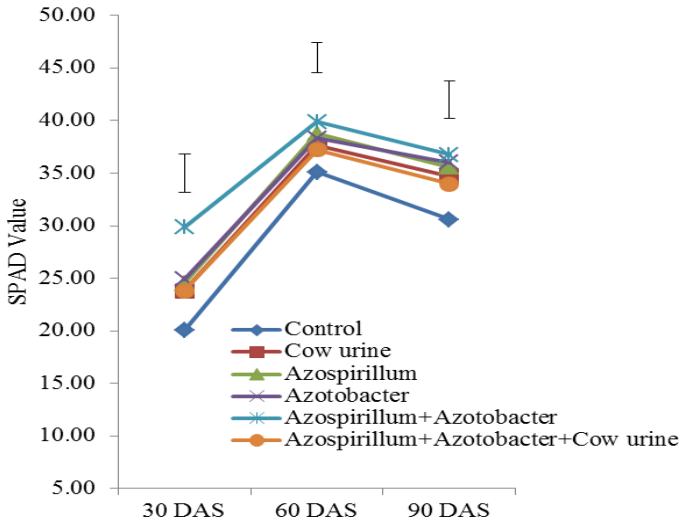

Figure 1. Inoculation effect on leaf chlorophyll content (SPAD) of common buckwheat (Mean Data of 2 years). The vertical bars indicate C.D. at *P* = 0.05.

Table 2. Inoculation effects on yield attributes and yield of common buckwheat (Mean data of 2 years).

Treatment	Seeds/plant	Seed yield/plant (g)	Test weight (g)	Yield (Mg/ha)
Control	101	2.02	21.5	0.95
Cow urine	102	2.23	22.4	0.99
Azospirillum	128	2.45	23.0	1.17
Azotobacter	111	2.13	22.4	1.04
Azospirillum+Azotobacter	135	2.99	23.7	1.23
Azospirillum+Azotobacter+Cow urine	121	2.65	22.6	1.05
SEm±	1.2	0.13	0.12	0.05
CD (*P*=0.05)	3.6	0.40	0.37	0.14

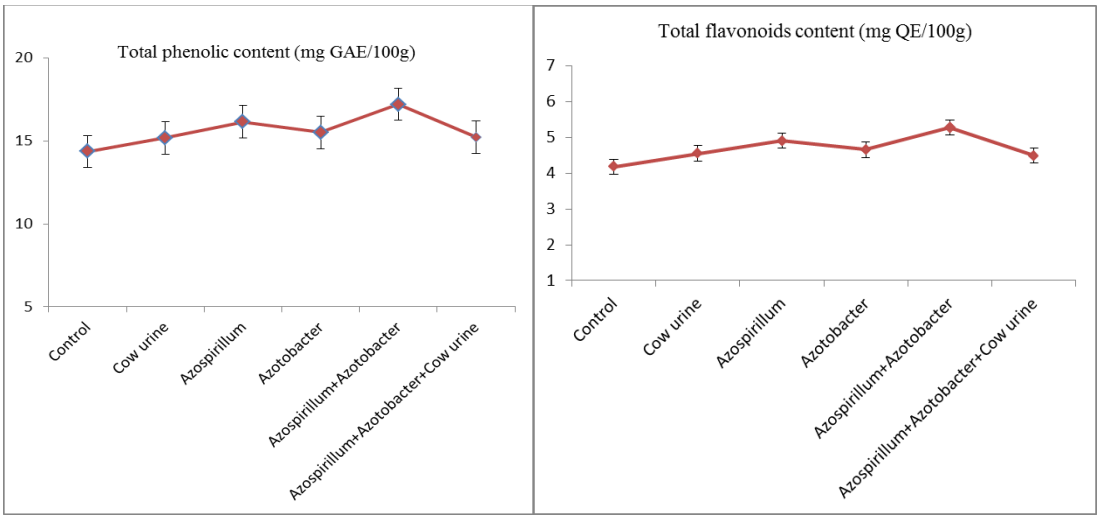

Figure 2. Inoculation effect on total phenolic (mg GAE/100g of seed) and total flavonoids content (mg QE/100 g seed) of common buckwheat (mean data of 2 years).The vertical bars indicate C.D. at *P* = 0.05.

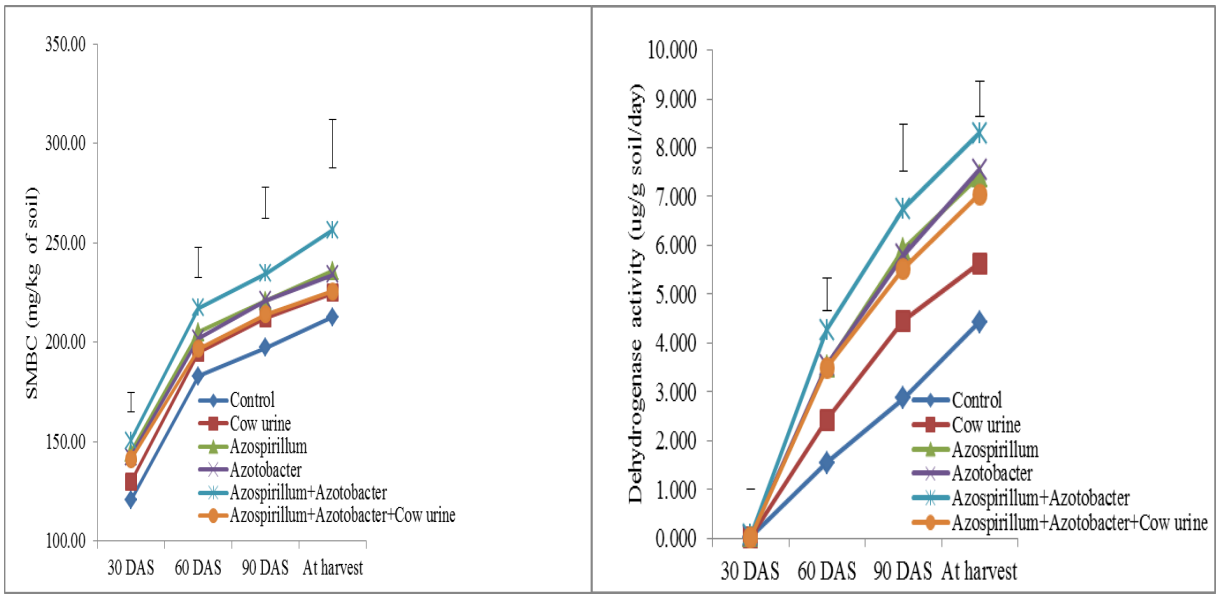

Figure 3. Inoculation effect on soil microbial biomass carbon (mg/kg of soil) and soil dehydrogenase activity (µg/g soil/day)of common buckwheat (mean data of 2 years). The vertical bars indicate C.D. at *P* = 0.05.

combined application of over the control, and other treatments during at all the growth stages *viz.,* 30, 60, 90 DAS and at harvest.

DISCUSSION

Growth of common buckwheat

Significant response in plant growth characteristic of common buckwheat plants was observed under inoculated plots compared to un-inoculated ones. Inoculation of *Azospirillum* spp. and *Azotobacter* spp. exerted the significant effect on all the growth parameters at all the growth stages of buckwheat over control. This was due to *Azospirillum* spp. and *Azotobacter* spp. playing pivotal role in nitrogen fixation which may improve the nitrogen fixation. In addition, they provide growth promoting substances, such as indole acetic acid and gibberellins (Fayez et al., 1985). Poor growth characteristics in control plots and higher growth in treated plots could be due to poor and higher nutrients supply, respectively. The positive effects of seed inoculation reflects on plant growth in this study have also been reported by Nwangburuka et al. (2012). They observed that inoculated plants grown with organic amendments produced higher growth characteristics than un-inoculated ones. Increase nutrients availability in soil due to biofertilizers were reported by several workers (Sridevi and Ramakrishnan, 2010; Geeta et al., 2013). In the study combined application of *Azospirillum* spp. +*Azotobacter* spp. resulted in maximum plant height, stem girth, leaves/plants, root length, root and top dry weight at all the stages of plant growth over the others. Better plant growth might be due to proper supply of nitrogen and growth promoting hormones by *Azospirillum* spp. + *Azotobacter* spp. and enhanced uptake of phosphorus and other nutrients due to mycorrhizal colonization (Zaidi et al., 2004). Enhanced nutrients availability could also be attributed to the decomposition of organic manure or transforming of inorganic substances to available form by microorganisms. These results are supported by the findings of Tao et al. (2004).

Chlorophyll content (SPAD), yield attributes and yields of common buckwheat

The results (Figure 2) show that the chlorophyll contents of common buckwheat are relatively lower in seeding stage but with time the chlorophyll contents increase, and reached the maximum when they are in full bloom stage (60 DAS), and thereafter the chlorophyll contents decline gradually. Seed inoculation recorded higher SPAD values at all the stages of crop growth over control. Across the growth stages, the combined application of by *Azospirillum* spp. + *Azotobacter* spp. recorded about 13-49% higher chlorophyll content (SPAD) over control. The beneficial effects of bacterial inoculation on increased chlorophyll content might be due to the higher amount of nitrogen supplied to the growing tissue and organs supplied by N_2 fixing *Azospirillum* spp. and *Azotobacter* spp.. When nitrogen levels in plant tissues are low, plants do not metabolize nutrients efficiently (Conley et al., 2002). According to Haboudane et al. (2002), the higher the SPAD value, the greater the chlorophyll and nitrogen

content of the leaves (Swiader and Moore, 2002). The increase in chlorophyll content with increasing nitrogen has also been reported by Seneweera et al. (2011). All yield attributes were found superior with seed inoculation as compared to control and this could be assigned to better growth and development of plants with higher dry matter accumulation, robust growth and increased photo-synthetic activity which resulted in higher accumulation of photosynthates. The number of leaves is an important factor, because the leaves are structures bearing photosynthetic machinery and an increase in leaf number may promote better root development, better translocation of water uptake and deposition of nutrients and yield (Chandrasekhar et al., 2005). Combined application of *Azospirillum* spp. and *Azotobacter* spp. resulted in maximum value of yield attributes among the treatments. Plant growth regulating substance such as indole acetic acid (IAA), gibberellic acid ($GA_{3)}$ and cytokines produced by *Azospirillum* spp. and *Azotobacter* spp. are known to promote better growth (Tiwary et al., 1998). The yield of the crop is final product of various yield attributing characters. The effect of any treatment on yield attributes is directly reflected in the yield. In this study, Combined inoculation of *Azospirillum* and *Azotobacter* recorded 29.5, 24.2, 5.1, 18.3 and 17.1 per cent higher yield over the control, cow urine treatment, *Azospirillum* spp., *Azotobacter* spp., *Azospirillum* spp. +*Azotobacter* spp. and *Azospirillum* spp. +*Azotobacter* spp. + Cow urine, respectively. The higher grain yield due to biofertilizers inoculation might be due to increase in plant height and total chlorophyll content and yield component. Similar findings were also reported by Tao et al. (2004) and Babu et al. (2014).

Total phenolic (TPC) and flavonoid contents (TFC) of buckwheat seed

Genotype is the primary determinant of the composition of secondary plant metabolites (TPC and TFC), although their expression is strongly influenced by environmental pressures of climate. Seed inoculation with *Azospirillum* spp. and *Azotobacter* spp. had a significant ($P \leq 0.05$) impact on the production of total phenolics and flavonoid production (Figure 2). These microorganisms can fix atmospheric nitrogen and supply it to plants as they synthesize several different phytohormones that can act like growth regulators and may have mechanisms for the solubilization of minerals, such as phosphorus which may become more readily available for plant growth and they may synthesize some less well characterized low molecular mass compounds or enzymes that can modulate plant growth and development (Glick, 1995; Hanan et al., 2008) and resulted in great enhancement effect on total phenolics, total flavonoids, compared to their conventionally grown counterparts. This might be mainly due to better nitrogen supply by the microorganisms. When nitrogen supply was better, improvements in both phenol and flavonoids content were also reported by Sene et al. (2001). They also found positive correlation in grain yield and the phenol pool of aerial parts.

Soil biological properties

The data on microbial activity in terms of dehydrogenase activity and soil microbial biomass carbon during crop growth period was recorded at 30, 60, 90 DAS and at harvest and presented in Figure 3, respectively. These activities provide the information about the microbial growth and development. Dehydrogenase activity was chosen as an index of microbial activity as it refers to group of mostly endo cellular enzymes, which catalyze oxidation of soil organic matter (Pascual et al., 1998). In the present study, higher values of dehydrogenase activity and soil microbial biomass carbon were observed with microbial inoculants. The combined inoculation of *Azospirillum* spp. and *Azotobacter* spp. in buckwheat seed resulted almost in double activities of dehydrogenase enzyme in soil. Similarly, across the growth stages about 18-25% higher SMBC was observed due to the same treatment over control. This might be due to better establishment of inoculated microorganism, which stimulates the indigenous microorganisms. Our results suggest that seed inoculation should also improve the soil fertility by increasing the biological activity of soil, which in turn reduce the fertilizer requirements. Indeed, these results are very desirable from economic and ecological point of view (Piotrowska et al., 2012). These results are in close conformity with those reported by Abdullahi et al. (2013).

Conclusion

Azospirillum spp. and *Azotobacter* spp. thrives well in acidic soils of Sikkim and their combined application resulted in better buckwheat productivity and positively influenced the soil biological properties. Hence, this combination may be recommended for obtaining good crop yield and sustaining soil health.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are thankful to the Director of ICAR Research Complex for NEH Region, Barapani, India and NICRA (National Initiatives of Climate Resilient Agriculture) for providing necessary facilities and financial support to conduct this research.

REFERENCES

Abdullahi R, Sheriff H H, Lihan S (2013). Combine effect of bio-fertilizer and poultry manure on growth, nutrients uptake and microbial population associated with sesame (*Sesamumindicum*L) in North-eastern Nigeria. IOSR J. of Envi. Sci. Toxi. Food Tech. 5: 60-65.

Babu Subhash, Kalita H, Singh Raghavendra, Gopi R, Kapoor C, Das SK (2014).Buckwheat (*Fagopyrum* spp..).*In:* Handbook on organic crop production in Sikkim. (*Eds.* R. K. Avasthe, YashodaPradhan and Khorlo Bhutia).Sikkim Organic Mission, Govt. of Sikkim and ICAR Research Complex, Sikkim Centre, Tadong, Gangtok, Sikkim. pp. 47-52.

Casida LEJ, Klen DA, Santro T (1964). Soil dehydrogenase activity. Soil Bio. Biochem.98: 371-376.

CGIAR (2013). Annual report of programme on climate change, agriculture and food security http://ccafs.cgiar.org/

Chandrasekhar B R, Ambrose G, Jayabalan N (2005). In fluence of biofertilizers and nitrogen source levels on growth and yield of Echinochloa frumentacea (Roxb.) Link. J. Agric. Tech. 1:223-234.

Conley ME, Paparozzi ET, Stroup WW (2002). Leaf anatomical and nutrient concentration responses to nitrogen and sulfur applications in poinsettia. J. Plant Nutr. 25:1773-1791.

Fayez M, Eman NF and Makbol HE (1985). The possible use of nitrogen fixing *Azospirillum* as biofertilizer for wheat plants. Egypt. J. Microbiol. 20: 199-206.

Geeta B, Patil HC, Lakshman Romana, Mirdhe, Agadi BS (2013). Effect of co- inoculation of AM fungi and two beneficial microorganisms on growth and nutrient uptake *Eleusiencoracana* Gaertn. (Finger millet). Asian J. P. Sci. Res. 3: 26-30.

Glick BR (1995). The enhancement of plant growth by free bacteria.Can. J. Microbl. 41: 109-117.

Gomez K A, Gomaz A A (1984). *Statistical procedures for Agricultural Research.* John Wiley & Sons, Singapore

Haboudane D, Miller JR, Tremblay N, Zarco-Tejada P, Dextraze L (2002). Integrated narrow-band vegetation indices for prediction of crop chlorophyll content for application to precision agriculture. Remote Sens Environ. 81:416-426.

Hanan AA, Taie R, El-Mergawi, Radwan S (2008) Isoflavonoids, Flavonoids, phenolic acids profiles and antioxidant activity of soybean seeds as affected by organic and bioorganic fertilization. American-Eurasian J. Agric. Environ. Sci. 4:207-213.

Joshi BD, Paroda RS (1991).Buckwheat in India. NBPGR, Shimla Sci. Monogr, 2:l17.

Kontturi M, Marjo K, Ketoja E (2004). Buckwheat cultivars in the north***In:*** Proceedings of the 9th International Symposium on Buckwheat, Prague 2004. pp. 496-498.

Krzysztof D, Danuta G, Monika K, Barbara P (2012). Influence of technological process during buckwheat groats production on dietary fibre content and sorption of bile acids. Food Res Inter. 47:279-283.

Numan N, Morgan, MA, Helihy M (1998). Ultraviolet absorbance (280 nm) of compounds related from soil during chloroform fumigation as an estimate of the microbial biomass. Soil Biol. Biochem. 30:1599-1603.

Nwangburuka CC, Olawuyi OJ, Oyekale K, Ogunwenmo KO, Denton OA, Nwanko E (2012). Growth and yield response of *Corchorusolitorius* in the treatment of Arbuscularmychorrhizae (AM), poultry manure (PM), combination of AM-PM and inorganic Fertilizer (NPK). Adv. in App. Sci. Res. 3:1466-1471.

Pascual JA, Hernandez T, Garcia C, Ayuso M (1998). Enzymatic activities in an arid soil amended with urban organic wastes: Laboratory experiment. Biol. Tech. 64*:* 131-138.

Piotrowska A, Dlugosz J, Zamorski R, Bogdanowicz P (2012). Changes in soil biological and chemical properties of an arable soil treated with the microbial biofertilizer UGmax. Pol. J. Environ. Stud. 21(2):455-463.

Pirogovskaya GV, Rusalovitch AM, Soroko VI, Sazonenko OP, Shakovets OE (2004). Efficiency of new forms of mineral fertilizers for field grown buckwheat on light textured soils Proceedings of the 9th International Symposium on Buckwheat, Prague 2004. pp. 470-74.

Sene M, Dore T, Christiane Gallet Jan (2001).Relationships between biomass and phenolic production in grain sorghum grown under different conditions. Agron. 93:49-54.

Seneweera S, Makino A, Hirotsu N, Norton R, Suzuki Y (2011). New insight into photosynthetic acclimation to elevated CO_2: The role of leaf nitrogen and ribulose 1,5-bisphosphate carboxylase/oxygenase content in rice leaves. Environ. Exp. Bot. 71:128-136.

Singleton VL, Orthofer R, Lamuela-Raventos RM (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent.Meth.inEnzymol. 299:152-178.

Sridevi S, Ramakrishnan K (2010). Effects of combined inoculation of am fungi and Azospirillum 0n the growth and yield of onion (Allium cepa L.). J. Phyt.Phytophysio. 2:88-90.

Swiader JM, Moore A (2002). SPAD - chlorophyll response to nitrogen fertilization and evaluation of nitrogen status in dryland and irrigated pumpkins. J. Plant Nutr. 25:1089-1100.

Tao Y, Shf Q, Zhangl X, Zhou Y (2004). Inoculation effect on growth and flavonoid content of tartary buckwheat in a field experiment. Fagopyrum 21:45-50.

Tiwary DK, Abuhasan MD, Chattopadhyay (1998). Studies on effect of inoculation with Azotobacter and Azospirillum on growth, yield and quality of Banana. Indian J. Agric. Sci. 42:235-240.

Vance ED, Brooks PC, Jenkinson DS (1987). An extraction method for measuring soil microbial biomass C. Soil Biol. Biochem.19: 703-707.

Zaidi A , Khan M S, Aamil M (2004). Bioassociative effect of rhizospheric microrganisms on growth , yield and nutrient uptake of green gram. J. Plant Nutr. 27:599-610.

Zhang X, Chai Y (2001). Effects of rates and combinations of applied nitrogen and phosphorus fertilizers on kernel protein components of buckwheat.Proc. 8th IntI. Symp. Buckwheat at Chunchon: 90-98.

Zhishen J, Mengcheng T, Jianming W (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. 64:555-559.

African Journal of Microbiology Research

Related Journals Published by Academic Journals

- *African Journal of Biotechnology*
- *African Journal of Biochemistry Research*
- *Journal of Bacteriology Research*
- *Journal of Evolutionary Biology Research*
- *Journal of Yeast and Fungal Research*
- *Journal of Brewing and Distilling*

academicJournals