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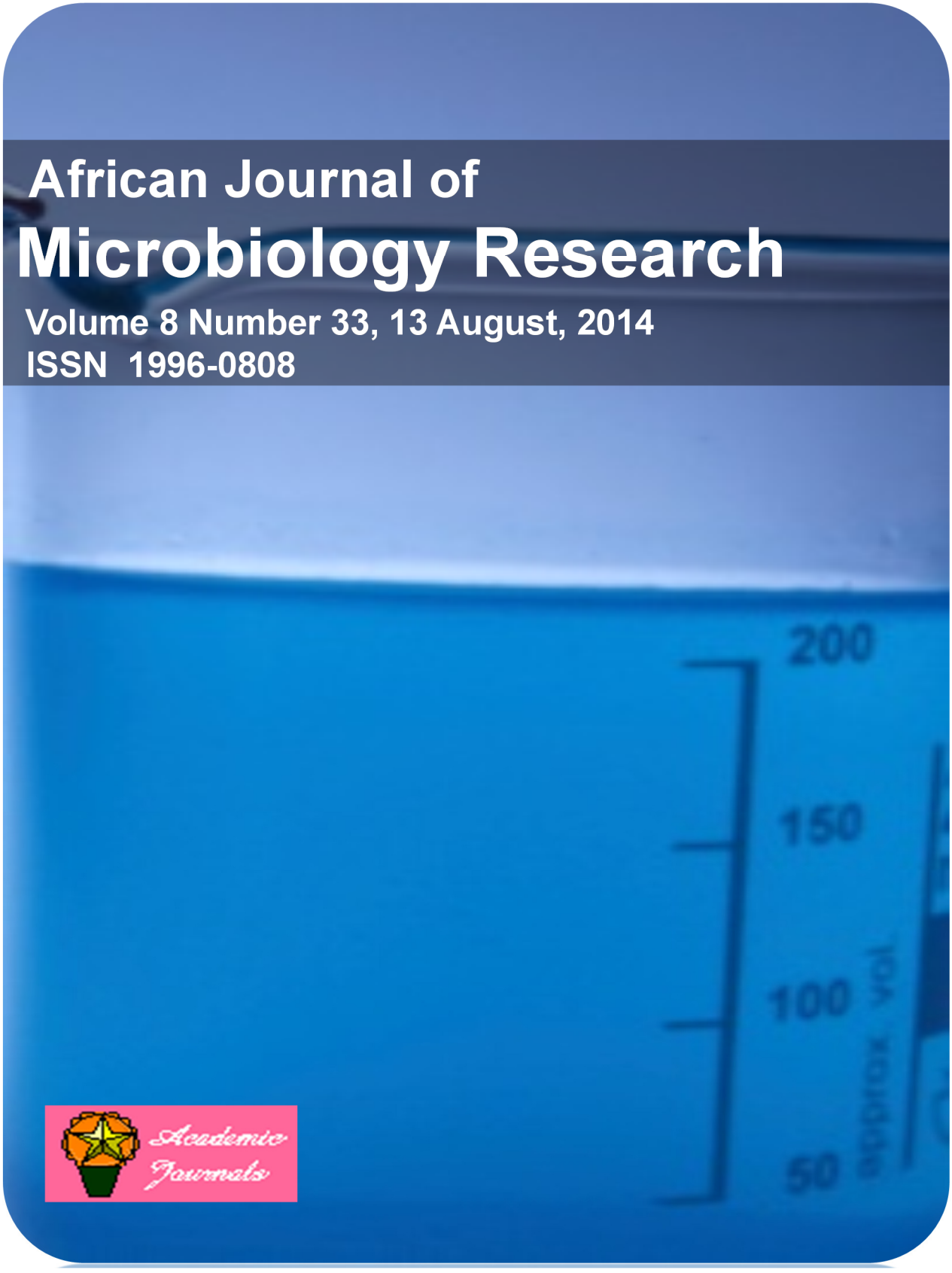

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: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

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Review

Biocontrol mechanisms by *Trichoderma* through genomics and proteomics analysis: A review

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Soil-borne phytopathogenic fungi pose serious threats to yield of several crops. Biological control is an eco-friendly approach in the effective management of crop diseases. *Trichoderma* is an important soil-borne fungus, which play an important role in antagonism by secretion of different hydrolytic enzymes. Lentil is an important pulse crop world-wide and its yield is severely affected by wilt disease. Plant pathological and molecular dissemination of the genus *Trichoderma* have reached the ultimate level of research and have been figured in almost all the pioneers of scientific publications. Enough of this exploration and exploitation! Now it is time to discover the *in silico* approach of this fungi. Many features and roles of this fungus are of biological importance that cannot be left unseen such as biological control mechanism, plant growth promoter, dying agent, etc. to name a few. Bioinformatics methods, tools and protocols, when applied to the genome of *Trichoderma* species, have provided many valuable and considerable results. Genomics tools and methods brought new genes that play important roles in mycoparasitism and biocontrol mechanisms for plant protection against pathogens. Open reading frames, promoter regions, gene-finding, primer designing, domain analysis, motif prediction, similarity searching in BLASTn, BLASTp, BLASTx have predicted some important regions of interest that have been found to be functional in the genome.

Key words: Genomics, *Trichoderma*, Mycoparasitism, cell wall degrading enzymes (CWDEs), bioagent.

INTRODUCTION

Many plant pathogens that cause major diseases in agricultural fields are controlled by the well-known biocontrol agent, *Trichoderma*. *Trichoderma* species are recognized for their production of enzymes called cell wall degrading enzymes (CWDEs) such as xylanase and glucanase (Pandey et al., 2014) that can be used for industrial production. All living organisms are made up of genes that code for a protein which performs the particular function. Some genes that play an important role in the biocontrol process are known as the biocontrol genes. These genes send some signals which help in secretion of proteins and enzymes that degrade the plant

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pathogens. These biocontrol genes can be cloned in huge quantities and can be used on large scale for commercial production (Massart and Jijakli, 2007).

Some *Trichoderma* genes are also helpful in providing resistance to the biotic and abiotic stresses such as heat, drought and salt (Kuc, 2001). The major biocontrol processes include antibiosis, mycoparasitism and providing plant nutrition (Janisiewicz and Korsten, 2002).

Trichoderma harzianum is the most effective strain among the various species of the *Trichoderma* used for biocontrol mechanisms (Gao et al., 2002).

The fungal pathogens were known to cause major diseases in the crops. So, most of the farmers were using hazardous chemical pesticides which cause major problems in the yield. *Trichoderma* species were involved in the biocontrol activity and their mechanisms of action were well known by the characterization and expression of their genes. This problem has been reduced by use of microbial biocontrol genes. With the help of genetic engineering techniques, more beneficial genes should still be discovered to develop the agriculture. Genes isolated from these biocontrol agents have been found to play an essential role in biocontrol activity. Chitinase, tubulins, protease, xylanase, galacturonase, glucanase, stress tolerant genes and cell adhesion proteins are the major kind of biocontrol genes (Pandey et al., 2014) that can be easily isolated, cloned and characterized. Cell wall degradation, hyphal growth, parasitic activity and stress tolerance are the major biocontrol mechanism by these genes.

ROLES OF BIOCONTROL GENES

The sequencing of fungal genomes is advancing at breakneck-speed, producing voluminous amounts of data. Within the next five years, it is possible that over a couple thousand genomes, representing every major fungal family will be completed and available to the scientific community. In order for this data to have a truly transformative effect on mycological and other research, several factors need to be addressed. These include: (1) the establishment of user friendly platforms for examining, sorting, and sifting through the genomes, (2) integration, or at least cross-communication, between the various databases that house the genomic data, and (3) investment in community resources that can act as repositories and provide materials to researchers, that is, strains, clones, plasmids, etc. The frameworks for some these needs, e.g. the materials available from the Fungal Genetics Stock Center (FGSC, University of Missouri), are already established and should be reinforced, whereas for others, e.g. data accessibility, the sooner a plan can be implemented, the better. The Fungal Kingdom is considered to contribute greater than 15% of the species richness found in the major groups of organisms. This study is a reflection of the usefulness of sequence analysis of the 28S ribosomal RNA gene in identifying fungal as well as determining fungal diversity. Various techniques that are based on utilizing the 28S rRNA have been discussed. Of critical importance is the manner in which massively parallel sequencing was exploited to correct the under representation of fungal species in compilations of fungal that were drawn using traditional methods of surveying fungal species from ecosystems (Srivastava et al., 2014).

GENES INVOLVED IN BIOCONTROL MECHANISMS

Degradation of fungal cell wall

The gene *Tvsp1* was cloned successfully from *Trichoderma virens,* its function was analyzed and it was proved to encode for serine protease. *Rhizoctonia solani* which affects the cotton seedlings has been controlled biologically by serine protease (Pozo et al., 2004).

The objectives of this research were to characterize isolates of *Trichoderma* collected from rhizospheres of chickpea, pigeonpea and lentil crop from different places of Uttar Pradesh, India, using microsatellite-primed polymerase chain reaction (MP-PCR) and ribosomal DNA (rDNA) sequence analysis and to combine these results with morphological characteristics for classification.

Thirty isolates of *T. viride* obtained from rhizosphere soil of plantation crops, and agricultural fields of UP region were studied using ISSR and ITS-PCR. The genetic relatedness among thirty isolates of *T. viride* was analyzed with six microsatellite primers. ISSR profiles showed genetic diversity among the isolates with the formation of eight clusters. Analysis of dendrogram revealed that similarity coefficient ranged from 0.27 to 0.95. ITS-PCR of rDNA region with ITS1 and ITS4 primers produced 600 bp products in all isolates. This result indicated the identification patterns of *Trichoderma* isolates (Shahid et al., (2014).

In *T. harzianum,* trichodiene syntheses gene *tri*5 was isolated and characterized. This *tri*5 gene is responsible for the synthesis of the enzyme trichothecene which inhibits the protein and DNA synthesis in the cells of the pathogens and inhibits their growth. The trichothecene shows phytotoxic activity against *Fusarium* species. The presence of *tri*5 gene was confirmed by screening with other *Trichoderma* isolates (Gallo et al., 2004). The enzyme activity of glucanase was studied by comparing with various types of carbon sources like starch, cellulose, chitin, chitosan and cell walls of *R. solani*. The expression of *tag83* gene with *R. solani* showed that glucanase enzyme exhibits parasitic activity against pathogens. The expression of gene *tag83* which encodes cell wall degrading enzyme exo-*β*-1,3-glucanase was isolated from *Trichoderma asperellum* and characterized. The expression analysis of this gene was studied using real time and reverse transcription-polymerase chain reac-

tion (RT-PCR) (Marcello et al., 2010). *T. virens* transformants expressed two different kinds of β-1,3 and β-1,6 glucanase genes viz., *TvBgn2* and *TvBgn3*. These genes secrete cell wall degrading enzyme that helps in the biocontrol activity. *T. virens* GV29.8 wild type and double over expression (DOE) transformant strains were used to detect the enzyme activity against pathogens like *R. solani*, *Pythium ultimum* and *Rhizopus oryzae* (Djonovic et al., 2007). *β*-Tubulins are structural components of most cells and they interact with benzimidazole fungicides, and play a major role in biocontrol process. This *β*-tubulin gene was isolated and characterized from *T. harzianum*. The *β*- tubulin gene was amplified by PCR, the coding regions and the flanking sequences were identified using inverse and nested PCR. The sequences were analyzed for the presence of motifs for the expression of the gene. The three dimensional model of *β*-tubulingene was done by Swiss-model automated comparative protein modeling server (Li and Yang, 2007). From *T. virens,* a gene, Sm1 a cysteine-rich protein was isolated and expressed. It shows defense activity against diseases in dicot and monocot plants (Buensanteai et al., 2010). From *Trichoderma atroviride* a gene *gluc78* which codes for an antifungal glucan 1,3-β-glucosidase was isolated, cloned and sequenced. This gene has its significance in the pathogen's cell wall degradation. The gene *gluc78* was cloned in pGEM-T vector and the expression analysis was done against pathogens such as *R. solani* and *P. ultimum* (Donzelli et al., 2001). From *T. harzianum*, a glucose repressor gene *creI* was isolated and characterized. This gene causes the repression of cellulase and xylanase encoding genes. Cellulase and xylanase are the major type of enzymes involved in the pathogen's cell wall degradation. The gene was cloned using pTZ57R/T plasmid vector and transformed into *E. coli* DH 10B and the role of *creI* gene in cellulase and xylanase expression was studied (Saadia et al., 2008). Serine proteases play a key role in the fungal biology and involves in biocontrol activity. From *T. harzianum* a novel serine protease gene named SL41 has been cloned and expressed successfully in *Saccharomyces cerevisiae*. The cDNA ofSL41 gene was sequenced and it was cloned in pMD18-T vector and the yields were inserted into *E. coli* DH5-α. Thus, serine proteases were cloned and characterized (Liu et al., 2009).

Xylanase producing *Trichoderma* strain SY was isolated from the soil. The gene coding for xylanase, *Xyl* was cloned by RT-PCR. *Xyl* was highly expressed when it was grown in cellulose as an only carbon source. The full length DNA of *Xyl* was amplified by PCR and cloned in pGEM-T vector. The cloned gene was expressed in *E. coli* and the proteins were analyzed using sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) (Min et al., 2002). From *T. virens*, the g-protein α subunits genes, *TgaA* and *TgaB* were cloned and characterized. This gene exhibits antagonist activity against *R. solani* and *Sclerotium rolfsii* (Mukherjee et al., 2004). The gene *ThPG1* was isolated from *T. harzianum,* characterized and proved to encode for endopolygalacturonase.

This enzyme is involved in the cell wall degradation of pathogens like *R. solani* and *P. ultimum*. The expression study of this gene was studied by comparing the wild and mutant type strains. The full length cDNA clone of *ThPG1* gene was obtained by polymerase chain reaction and was cloned in pSIL-pG1 vector. The phylogenetic relationship was obtained by neighbor-joining (NJ) tree method (Moran Diez et al., 2009). A gene, *Tv6Gal* which codes for endo-*β*-(1→6)-galactanase gene was isolated from *T. viride*, cloned and expressed in *E. coli*. Galactanase enzymes belong to the family of arabinogalactan proteins involved in cell-cell adhesion, cell expansion and cell death. The cDNAclone of the gene *Tv6Gal* was done by RT-PCR, cloned in pGEM-T vector and expressed in *E. coli* (Kotake et al., 2004).

Antifungal activity

Endochitinase gene named *Th-Chit* was isolated from *T. harzianum* that is responsible for the antifungal activity in transgenic tobacco plant. The gene *tri5 from T. brevicompactum* encodes a trichodiene syntheses. The over expression of this gene helps in the production of trichodermin which shows antifungal activity against *S. cerevisiae, Kluyveromyces marxianus, Candida albicans, Candida glabrata, Candida tropicalis* and *Aspergillus fumigates.*

The *erg1* gene from *T. harzianum* was cloned and characterized. This gene encodes an enzyme named squalene epoxidase, which helps in the synthesis of ergosterol and silencing of this gene provides resistance to terbinafine, an antifungal compound. The antifungal activity was checked with *S. cerevisiae*. pSIL-E1 vector was used to clone the gene *erg1*. This is the first terpene biosynthesis gene characterized from *Trichoderma* genus (Cardoza et al., 2006). From *T. hamatum,* monooxygenase gene was isolated and characterized. This gene helps in the antifungal activity against some pathogens like *Sclerotinia sclerotiorum*, *Sclerotinia minor* and *Sclerotium cepivorum.* The expression of monooxygenase gene was influenced by the two fungal species contact, and its expression seems to be particularly important at pH 4.

The morphological, physiological, molecular characterization and bio-formulation of *T. harzianum* (Th Azad) and *T. viride* 01PP-8315, an effective fungicide and a biological control agent that protects the plants and seeds from other pathogenic fungi, have been investigated. The physiologic study is done in an attempt to find the effective management of the disease caused by soil borne pathogens. *T. harzianum* (Th Azad) and *T. viride* 01PP-8315 were isolated from the infected field's soil samples of pigeon pea grown at preferable temperatures,

pH and from different solid and liquid culture media. Most preferable temperature for the growth and sporulation of *T. harzianum* (Th Azad) and *T. viride* 01PP-8315 has been observed to be up to 30°C (210.5 mg dry weight of mycelium). A detailed morphology of the strain is described in this study including colony growth rate, colony color, colony edge, mycelial form, conidiation, conidiophore branching, conidial wall, conidial color, etc. The molecular characterization of the strain is carried out using an 18S rRNA gene sequence with the help of a universal Internal Transcribed Spacer marker that gives an amplicon of a total of 1173 base pairs. The 546 pb 18S rRNA gene was sequenced and used for the identification of isolated fungal strains that is later sequenced and allotted with Gene Bank Accession no. JX119211 and KC800922, respectively. To check the presence of endochitinase gene in two of the potential strains of *Trichoderma* species viz. *T. harzianum* (Th Azad) and *T. viride,* 01PP an ech42 primer was used. A talc based bioformulation has been prepared with this strain where the population of the spores was found to decline after 180 days (Shahid et al., 2014).

STRESS TOLERANCES: BIOTIC AND ABIOTIC

Trichoderma species helps the plant to survive in the abiotic stress conditions. From *T. virens* glutathione transferase gene *TvGST* was cloned. When transgenic plant expresses this gene against different concentrations of cadmium, it shows tolerance to cadmium accumulation in plants. Thus it acts as cadmium tolerant gene (Dixit et al., 2011). From *T. harzianum* T34 isolate, *hsp*70 gene was cloned in pGEM-T vector, expressed in different isolates of *T. harzianum* and characterized as a gene that helps in increasing the fungal resistance to heat and salt tolerance, osmotic and oxidative tolerances. The protein sequences were analyzed using DNA star package and aligned using CLUSTAL X algorithm (Mantero Barrientos et al., 2008).

A gene named *Thkel1* was isolated and characterized from the fungus *T. harzianum*. This gene codes for putativekelch-repeat protein which helps in regulating the glucosidase activity and enhances tolerance to salt and osmotic stresses in *Arabidopsis thaliana* plants. The vector used for cloning was p*SIL-KEL* and was transformed to *T. harzianum.* The expression of this gene was studied by growing the fungal at various biotic and abiotic stress conditions (Hermosa et al., 2011).

Mycoparasitic action

Genes were cloned and expressed from five isolates of *T. harzianum* namely (T 30, 31, 32, 57 and 78) encoding for *N-acetyl-β-D-glucosaminidase (exc1* and *exc2),* chitinase (*chit*42 and *chit*33), protease (*prb*1) and β-glucanase (*bgn* 13.1).

These genes play a major role in the mycoparasitic activity against the pathogens especially *Fusarium oxysporum*. The expressions of these genes that codes for these enzymes were determined by RT-PCR and their effects against the pathogens were tested by dual plate assay.

Trichoderma longibrachiatum transformants showed over expression of β-1,4-endoglucanase gene *egl1*. This gene showed biocontrol activity against *P. ultimum* in cucumber's damping- off. The *egl1* gene, coding for endoglucanase was isolated from *T. longibrachiatum*, cloned and expressed in *S. cerevisiae.* The expression of the gene was compared with the wild type and transformed strains. The results showed that the over expression of *egl1* gene showed good biocontrol activity (Migheli et al., 1998).

The gene, *qid74* isolated from *T. harzianum* CECT 2413 was found to play a significant role in cell protection and provide adherence to hydrophobic surfaces that helps the fungus in mycoparasitic activity against *R. solani* pathogen. The function of this gene was studied by comparing the expression of genes in wild type transformants and disruptants.

A gene named, *Taabc2* was cloned from *T. atroviride* and characterized. This gene has a significant role in ATP binding cassette (ABC) transporter in cell membrane pump that helps in the mycoparasitic activity. The gene was cloned using pGEM-T vector and their expression was analyzed using RT-PCR. The antagonist activity against pathogens such as *R. solani*, *Botrytis cinerea*, and *P. ultimum* was done by dual culture plate assay with *T. atroviride* wild and mutant type strains (Ruocco et al., 2009).

In *T. virens*, an adenalyte-cyclase encoding gene named *tac1* gene was isolated and cloned. This gene has its role in mycoparasitic activity against *R. solani* and *P. ultimum* (Mukherjee et al., 2007). *ThPTR2* a di or tri peptide transporter gene isolated from *T. harzianum* CECT 2413 has a significant role in the mycoparasitic activity against *B. cinerea.*

Genes responsible for hyphal growth

A new gene, named *TrCCD1* was isolated from *Trichoderma reesei*. This gene is involved in carotenoid metabolism that helps in the development of conidiospores and hyphal growth in *T. reesei*. The function of the gene was analyzed by comparing two mutant types named *ccdO* and *ccdP* (carotenoid cleavage dioxy- genase) with the parental type. The T-DNA insertion of fungal genome was sequenced using specific primer, multiple sequence alignment was done using CLUSTAL- W algorithm and phylogenetic relationship was done by neighbor joining method (Zhong et al., 2009).

BIOINFORMATIC APPROACHES

Proteomic techniques are able to separate and characterize complex sets of proteins. Moreover, the vast majority of current drug targets are proteins. As well as using proteomics to settle drug targets of a specific compound or to search new therapeutics objectives, the development of mathematical algorisms to predict its role may be a useful tool for drug discovery through proteomics analysis. Xu et al. (2007) presented the concept of "drug target-likeness" of a protein as an independent set of characteristics of successful targets. By a thorough study of known drug targets, it is possible to determine if an obtained protein sequence fits with this drug target role. This methodology may open a new frontier in fungicide design, algorithm applies to fungal plant pathogens, accumulation of molecular information on these organisms, and commercial interest of developing environmentally friendly fungicides, will press the research community to improve mathematical algorisms to predict the role of a protein as a fungicide. Proteomics methods helped in finding the functional peptides that show significant functions in the biocontrol mechanism. These peptides have successfully been identified in the peptide mass fingerprinting analysis through MALDI-TOF mass spectrometry. *T. viride* and *T. harzianum* consist of numerous peptides that are clearly visible in the mass spectra. Cell wall degrading enzymes such as chitinase, glucanase, proteinase and xylanase have been identified in the *Trichoderma* species under consideration and protein structural determination of the modeled structures (in UCSF Chimera) bring in front the N- and C-termini and the active sites of the proteins.

New protein-based strategies to classical chemical fungicide design

Historically, drugs have been obtained from plant and animals products, from derivates of human endogenous legends or from chemicals or semi-synthetic chemicals. Classical methods to control fungal plant diseases are based on the use of chemical compounds. In spite of the success achieved, new criteria for the indiscriminate use of toxic compounds in nature avoid using this technology. Control strategies based on classical fungicides produce serious collateral effects, mainly related with environmental pollution and the development of multidrug resistance.

Several changes in the design of chemical fungicides are being tackled by the research community by summarizing the genomic and proteomic information available. Biosynthetic fungicide design has been established as a new focus in fungicide development (Collado and Sanchez, 2007). Based on a deep study of fungal biology, the use of alien or modified natural compounds provides a potential species-specific method of controlling plant pathogens by specific inhibition of those proteins involved in the infection cycle (Pinedo et al., 2008). The use of these compounds minimizes the environmental impact as they are biodegradable, possess high specificity, and integrate poorly in the food chain.

In the post-genomic era, new terms related with chemical "-omics" have appeared. The term "genetic chemical" describes the use of small molecules that selectively perturb gene function. When this concept is applied on a genome-wide scale it is named "chemogenomics". The application of chemogenomics to protein targets is named "chemoproteomics"; although a more explicit definition is targeted related affinity profiling (TRAP) defined as the use of biology to inform chemistry (Beroza, 2002). The accumulation of proteomic information of fungal plant pathogens may be an incentive to the development of new and environmentally friendly fungicides.

Conflict of Interests

The authors have not declared any conflict of interests.

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Review

Halophilic microbes for bio-remediation of salt affected soils

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Bacteria inhabiting soil play a role in conservation and restoration biology of higher organisms. The salt affected soils are dominated by many types of halophilic and halotolerant microorganisms, spread over a large number of phylogenetic groups. These microbes have potential for plant growth promotion and release of enzymes under salt stress. Halophiles have thus potential to remediate salt affected soils, enhancing plant growth and yield under high salt containing soils where plant growth is restricted. This eco-friendly approach for bio-remediation of salt affected soils to optimize crop yields under stress through halophiles has gained importance among researchers in recent past.

Key words: Halophiles, bio-remediation, saline soils, salt tolerant bacteria.

INTRODUCTION

Life exists over the whole range of salt concentrations encountered in natural habitats: from freshwater environments to hypersaline lakes or seas, and other places saturated with respect to sodium chloride especially saline and saline-alkali soils. In many cases, the soil properties are the most definitory of the limitations for the ecosystem functioning and, quite always, the soil is the component of the ecosystem more resilient to changes. The influence of the high salt concentrations masks other soil forming processes or soil properties and environmental conditions, often altering them.

The soil that contains excess salts which impairs its productivity is called salt-affected (Figure 1) (Chhabra, 1996). Salt accumulation in soil is characterized by saline soil, contains high amount of soluble salts Ca^{2+}, Mg^{2+}, K^{+} and Na^{+} salt of Cl^{-}, NO_3^{-}, SO_4^{2-} and CO_3^{3-} etc; Sodic soil, dominated by Na^{+} salt and saline-sodic soil that have high salt of Ca^{2+}, Mg^{2+} and K^{+} as well as Na^{+}. Salt-affected soils occupy an estimated 952.2 million ha of land in the world which constitutes nearly 7% of the total land area and nearly 33% of the area of potential arable land. In India, the salt affected soils account for 6.727 million ha (2.1%) of geographical area of the country. A build-up of soluble salts in the soil may influence its behaviour for crop production through changes in the proportions of exchangeable cations, soil reaction, physical

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Figure 1. Salt affected soils.

properties and the effects of osmotic and specific ion toxicity.

Physical and chemical methods of their reclamation are not cost-effective and also the availability of gypsum or other chemical amendments is a problem. The applications of halophilic bacteria include recovery of salt affected soils by directly supporting the growth of vegetation thus indirectly increasing crop yields in salt affected soils. All halophilic microorganisms contain potent transport mechanisms, generally based on Na^+/H^+ antiporters, to expel sodium ions from the interior of the cell (Oren, 2002). Also, some halophiles express ACC deaminase activity that removes stress, ethylene from the rhizosphere and some produce auxins that promote root growth. Halophilic microbes are also found to remove salt from saline soils (Bhuva et al., 2013).

There are reports that potential salt tolerant bacteria isolated from soil or plant tissues and having plant growth promotion trait, helps to alleviate salt stress by promoting seedling growth and increased biomass of crop plants grown under salinity stress (Ravikumar et al., 2007; Chakraborty et al., 2011).

Endophytic salt tolerant bacteria residing within plant tissues have been reported to be promoting the plant growth directly or indirectly through production of phyto-hormones, bio-control of host plant diseases and improvement of plant nutritional status (Pandey et al., 2008; Rosenblueth and Romero, 2006; Arora et al., 2014). They also possess the capacity to solubilize phosphates (Hung and Annapurna, 2004; Son et al., 2006).

Plant-microbe interaction is beneficial association between plants and microorganisms and also a more efficient method used for the reclamation of salt affected soils. Bacteria are the most commonly used microbes in this technique. However, archaea, fungi, actinomycetes, etc that may be found in environments with high salt concentrations can also be effective. Thus, there is high potential for bio-remediation applications for salt affected soils using halophilic microbes.

Halophilic microbes

The existence of high osmotic pressure, ion toxicity, unfavourable soil physical conditions and/or soil flooding, are serious constraints to many organisms and therefore salt-affected ecosystems are specialised ecotones. The organisms found there have developed mechanisms to survive in such adverse media, and many endemisms. The halophilic microorganisms or "salt-loving" micro-organisms live in environments with high salt concen-tration that would kill most other microbes. Halotolerant and halophilic microorganisms can grow in hypersaline environments, but only halophiles specifically require at least 0.2 molar (M) of salt for their growth. Halotolerant microorganisms can only tolerate media containing <0.2 M of salt. Distinctions between different kinds of halophilic microorganisms are made on the basis of their level of salt requirement and salt tolerance. According to Kushner (1993) classification, microbes responsive to salt are defined under five groups:

1. Non-halophilic, <0.2 M (~1%) salt,
2. Slight halophiles, 0.2-0.5 M (~1-3%) salt,
3. Moderate halophiles, 0.5-2.5 M (~3-15%) salt,
4. Borderline extreme halophiles, 1.5-4.0 M (~9-23%) salt, and
5. Extreme halophiles, 2.5-5.2 M (~15-32%) salt.

The halotolerant grow best in media containing <0.2 M (~1%) salt and also can tolerate high salt concentrations. This definition is widely referred to in many reports (Arahal and Ventosa, 2002; Ventosa et al., 1998; Yoon et al., 2003).

Soil microbial diversity

Microbial community in the soil are not distributed at random. Factors such as soil composition, organic matter, pH, water and oxygen availability, along with the

host plant, play major role in the selection of the natural flora (Ross et al., 2000). The soil gains importance, especially in saline agricultural soils, where high salinity results from irrigation practices and application of chemical fertilizer. This effect is always more pronounced in the rhizosphere as a result of increased water uptake by the plants due to transpiration. Hence, the rhizobacteria form a group of the best adapted microorganisms (Tripathi et al., 1998).

Saline or hypersaline soils have yielded many Gram-positive species, and these have been characterized taxonomically. The microbiota of hypersaline soils is more similar to those of nonsaline soils than to the microbiota from hypersaline waters. This suggests that general features of the environments are more important in determining the microbiota in a particular habitat than are individual factors such as high salinity (Quesada et al., 1983).

The structure and function of plant root system contributes to the establishment of the rhizosphere microbial population (Russell, 1977) and rhizosphere microbial communities are mainly determined by plant species (Marshner et al., 2001; Miethling et al., 2000) and soil characteristics (Degens et al., 2000). The microbial community found around the roots of plants or rhizosphere is generally much greater than in the surrounding soil environment (Nihorimbere et al., 2011). It reflects that the rhizosphere supports higher microbial growth rates and activities as compared to the bulk soil. One of the main reasons for these higher growth rates is the increased availability of soluble organic compounds that are the results of plant root exudation. These are typically carbohydrate monomers, amino acids and sugars, but the composition and quality of root exudates varies, depending on plant species and abiotic conditions such as water content and temperature. The organisms most commonly found in environments with high salt concentrations are bacteria, archaea, fungi, viruses, actinomycetes and vesicular arbuscular mycorrhiza (VAM).

Industrial biocatalysis is found in the halophilic micro-organisms, a source of enzymes with novel properties of high interest. Over the years, different enzymes of halotolerant and halophilic micro-organisms isolated from saline soils have been described and a number of new possibilities for industrial processes have emerged due to their overall inherent stability at high salt concentrations. These enzymes could be used in harsh industrial processes such as food processing, biosynthetic processes and washing (Ventosa et al., 2005).

Halopholic enzymes are active and stable at high salt concentrations, showing specific molecular properties that allow them to cope with osmotic stress. Mevarech et al. (2000) showed that these enzymes present an excess of acidic residues over basic residues and a low content of hydrophobic residues at their surface. Compatible solutes are low-molecular weight organic compounds such as polyols, amino acids, sugars and betaines that the halophilic and halotolerant bacteria accumulate intracellularly to achieve osmotic balance. On the other hand, halophilic bacteria tolerant to heavy metals could be used as bioassay indicator organisms in saline-polluted environments. Several halotolerant and halophilic bacteria isolated from hypersaline soils tolerate high concentrations of different metals such as Co, Ni, Cd, or Cr (Nieto et al., 1989; Rios et al., 1998).

In the biological treatment, the micro-organisms conventionally used show only poor degradative efficacy due to the highly saline conditions. The potential of halophilic organisms in effluent treatment offers the promise of innovative research. Other than that, halophilic is also used to recover saline soil by directly supporting the growth of vegetation, thus indirectly increasing crop yields in saline soil.

DIVERSITY OF HALOPHILIC SOIL MICROORGANISMS

Halophilic soil bacteria

The soil is an important habitat for bacteria. Soil bacteria can be found as single cells or as microcolonies, embedded in a matrix of polysaccharides. Bacteria inhabiting soil play a role in conservation and restoration biology of higher organisms. The domain bacteria contain many types of halophilic and halotolerant micro-organisms, spread over a large number of phylogenetic groups (Ventosa et al., 1998). The different branches of the Proteobacteria contain halophilic representatives often having close relatives that are non-halophilic. Similarly, halophiles are also found among the cyano-bacteria (Oren, 2002), the Flavobacterium - Cytophaga branch, the Spirochetes and the Actinomycetes. Within the lineages of Gram positive bacteria (*Firmicutes*), halophiles are found both within the aerobic branches (*Bacillus* and related organisms) as also within the anaerobic branches.

In general, it may be stated that most halophiles within the domain bacteria are moderate rather than extreme halophiles (Table 1). However, there are a few types that resemble the Archaeal halophiles of the family *Halobacteriaceae* in their salt requirements and tolerance.

Rodriguez-Valera (1988) stated that there was an abundance of halophilic bacteria in saline soil and that the dominant types encountered in saline soil belong to genera of *Alcaligenes*, *Bacillus*, *Micrococcus* and *Pseudomonas*. Garabito et al. (1998) isolated and studied 71 halotolerant Gram-positive endospore forming rods from saline soils and sediments of salterns located in different areas of Spain. These isolates were tentatively assigned to the genus *Bacillus*, and the majority of them were classified as extremely halotolerent

Table 1. Moderately halophilic bacteria.

Species	Gram's nature	Isolation Source	Reference
Bacillus krulwichiae	P	Soil from Tsukuba, Ibaraki,Japan	Yumoto et al. (2003)
Bacillus haloalkaliphilus	P	Showa, Saitama	Akinobu Echigo et al. (2005)
Bacillus oshimensis	P	Soil from Oshymanbe, Oshima, Hokkaido, Japan	Yumoto et al. (2003)
Bacillus patagoniensis	P	Rhizosphere of the perennial shrub Atriplex lampa in north-eastern Patagonia, Argentina	Olivera et al. (2005)
Gracilibacillus halotolerans	P	Shiki, Saitama	Akinobu Echigo et al. (2005)
Halobacillus halophilus	P	Salt marsh and saline soils	Spring et al. (1996)
Halobacillus karajensis	P	Saline soil of the Karaj region, Iran	Amoozegar et al. (2003)
Halomonas anticariensis	N	Soil from Fuente de Piedra. Málaga, Spain	Martínez-Cánovas et al. (2004)
Halomonas boliviensis	N	Soil around the lake Laguna Colorada, Bolivia	Quillaguaman et al. (2004)
Halomonas maura	N	Soil from a solar saltern at Asilah, Morocco	Bouchotroch et al. (2001)
Halomonas organivorans	N	Saline soil from Isla Cristina, Huelva, Spain	Garcia et al. (2004)

microorganisms, being able to grow in most cases in up to 20 or 25% salts.

Several alkaliphilic *Bacillus* species have been isolated from soil samples and it showed halophilic characteristics. *Bacillus krulwichiae,* a facultatively anaerobic (Yumoto et al., 2003), isolated in Tsukuba, Japan, is a straight rod with peritrichous flagella that produces ellipsoidal spores. These have ability to utilize benzoate or *m*-hydroxybenzoate as the sole carbon source. *Bacillus patagoniensis* (Olivera et al., 2005) was isolated from the rhizosphere of the perennial shrub *Atriplex lampa* in north-eastern Patagonia. Another is *Bacillus oshimensis* (Yumoto et al., 2005). It is a halophilic nonmotile, facultatively alkaliphilic species. Another example is the genus *Virgibacillus*. This genus comprises eight species, two of which are moderately halophilic and have been isolated from soil samples: *Virgibacillus salexigens* (Heyrman et al., 2003) and the recently described *Virgibacillus koreensis* (Lee et al., 2006).

Several other aerobic or facultatively anaerobic, moderately halophilic, endospore-forming, Gram-positive bacteria have been classified within genera related to *Bacillus*. Genera that include halophilic species isolated from soil samples are *Halobacillus*, *Filobacillus, Tenuibacillus*, *Lentibacillus* and *Thalassobacillus*. Species from *Filobacillus*, *Thalassobacillus* and *Tenuibacillus* genera are borderline halophile.

The genus *Halobacillus* is clearly differentiated from other related genera on the basis of its cell-wall peptidoglycan type. Within these genera, the halophilic species isolated from soils are: *Halobacillus halophilus* (Spring et al., 1996), *Halobacillus karajensis* (Amoozegar et al., 2003). With respect to the genus *Lentibacillus*, two halophilic soil species are identified. A *Lentibacillus salicampi* isolated from a salt field in Korea (Yoon et al., 2002), and A *Lentibacillus salarius* from a saline sediment in China (Jeon et al., 2005).

The family *Nocardiopsaceae* contains three genera, namely *Nocardiopsis* (Meyer, 1976), *Thermobifida* (Zhang et al., 1998) and *Streptomonospora* (Cui et al., 2001). Some examples of moderately halophilic species of the genus *Nocardiopsis* isolated from soil samples are: *Nocardiopsis gilva*, *Nocardiopsis rosea*, *Nocardiopsis rhodophaea*, *Nocardiopsis chromatogenes*, and *Nocardiopsis baichengensis* (Li et al., 2006). These all were isolated from saline sediment from Xinjiang Province, China.

From salt pans of Kovalam in Kanyakumari district of Kerala, India, Gram negative moderately halophilic bacteria like *Natranobacterium sp-1.* was identified in the study of the diversity over a period of time (Saju et al., 2011).

Many Gram-negative, moderately halophilic, or halotolerant species are currently included in the family *Halomonadaceae*. This family includes three genera with halophilic species: *Halomonas*, *Chromohalobacter* and *Cobetia*. Among the genera that comprise this family, *Halomonas* covers more than 40 species having heterogeneous features. Some species were isolated from soil samples: *Halomonas organivorans*, these species originating from saline soil samples in Spain, *Halomonas boliviensis* (Quillaguaman et al., 2004) were described as alkaliphilic and alkalitolerant, moderately halophilic bacteria, respectively, in as much as these bacteria are able to grow in media with pH values of about 8 to 9.

The genus *Marinobacter*, with the species *Marinobacter hydrocarbonoclasticus*, was created in 1992 to accommodate Gram-negative, moderately halophilic, aerobic Gammaproteobacteria that utilize a variety of hydrocarbons as the sole source of carbon and energy (Gauthier et al., 1992). It also accommodates moderately

halophilic *Marinococcus halophilus* and *Marinococcus albus* (Hao et al., 1984). Li et al. (2005) described a third species, *Marinococcus halotolerans* that is extremely halophilic. They are motile cocci that grow over a wide range of salt concentrations and up to 20% NaCl. The genus comprises 13 species, some of which are moderately halophilic bacteria isolated from soil samples: *Marinobacter lipolyticus*, that shows lipolytic activity with potential industrial applications (Martin et al., 2003), *Marinobacter excellens* (Gorshkova et al., 2003), *Marinobacter sediminum* (Romanenko et al., 2005), and the recently described *Marinobacter koreensis* (Kim et al., 2006).

Fungal diversity in saline environment

The importance of halophilic fungi, long neglected as members of hypersaline ecosystems, became recognized only in the past decade. Gunde-Cimerman (2009) (Ljubljana, Slovenia) gave an overview of the biology of the most widespread and most halophilic or halotolerant fungi and yeasts. These include the black yeasts *Hortaea werneckii* which grows up to 5 M NaCl, the true halophile *Wallemia ichthyophaga* that requires at least 1.5 M NaCl and grows up to saturation, and *Aureobasidium pullulans* that grows up to 3 M NaCl. According to Gunde-Cimerman et al. (2009), the recent definition of halophilic fungi are those species which are isolated regularly with high frequency on selective saline media from environments at salinities above 10% and are able to grow *in vitro* on media with at least 17% NaCl.

All of these are commonly found in hypersaline lakes and a great variety of other, often unexpected, environments: domestic dishwashers, polar ice, and possibly even on spider webs in desert caves (Gunde-Cimerman et al., 2009). In fungi, a low osmotic potential decreases spore germination and the growth of hyphae and changes the morphology (Juniper and Abbott, 2006) and gene expression (Liang et al., 2007), resulting in the formation of spores with thick walls. Fungi have been reported to be more sensitive to osmotic stress than bacteria. There is a significant reduction in the total fungal count in soils salinized with different concentrations of sodium chloride.

The halophilic and halotolerant fungi use polyols such as glycerol, erythritol, arabitol and mannitol as osmotic solutes and retain low salt concentrations in their cytoplasm. Molecular studies on osmotic adaptation of *Hortaea werneckii* and *Wallemia ichthyophaga* were presented by Plemenitas et al. (2014) (Ljubljana, Slovenia). Identification and structural features of Na^+-sensitive 3'-phosphoadenosine-5'-phosphatase HwHal2, one of the putative determinants of halotolerance in *H. werneckii* and a promising transgene to improve halotolerance in crops, was presented (Vaupotic et al., 2007). An in-depth understanding has been obtained on the high osmolarity glycerol (HOG) pathway, and this understanding may be applied in the future on the development of improved salt-resistant crops.

Yeasts and other fungi are chemoheterotrophic cell-walled eukaryotes, some of which are well adapted to tolerate hypersaline environments. They grow best under aerobic conditions on carbohydrates at moderate temperatures and acidic to neutral pH. *Debaromyces hansenii* is a halotolerant yeast, isolated from sea water, that can grow aerobically in salinities of up to 4.5 mol/L NaCl. It produces glycerol as a compatible solute during the logarithmic phase and arabitol in the stationary phase.

A saprophytic hyphomycete, *Cladosporium glycolicum*, was found growing on submerged wood panels at a salinity exceeding 4.5 mol/L NaCl NaCl in the Great Salt Lake. Halophilic fungi, e.g. *Polypaecilum pisce* and *Basipetospora halophila*, have also been isolated from salted fish (DasSarma and Arora, 2001).

Vesicular arbuscular mycorrhiza

VA-mycorrhizal fungi occur naturally in saline environment (Khan and Belik, 1994). Several researchers investigated the relationship between soil salinity and occurrence of mycorrhizae on halophytes. They reported that the number of VAM spores or infectivity of VAM fungi changed with change in salt concentration (Juniper and Abbott, 1993). The stresses due to saline soils effect the growth of plants, fungus or both.

VA-mycorrhizal fungi most commonly observed in saline soils are *Glomus* spp. (Juniper and Abbott, 1993) this suggest that this may be adapted to grow in saline conditions, but ecological specificity has not been demonstrated. There is evidence that VAM species distribution is markedly changed with increased salinity (Stahl and Williams, 1986).

Aliasgharzadeh et al. (2001) observed that the most predominant species of arbuscular mycorrhizal fungi (AMF) in the severely saline soils of the Tabriz plains were *Glomus intraradices*, *G. versiform* and *G. etunicatum*. The authors also found that the number of AMF spores did not significantly decrease with soil salinity and reported a relatively high spore number (mean of 100 per 10 g soil). The higher fungal spore density in saline soils may be due to the fact that sporulation is stimulated under salt stress (Tressner and Hayes, 1971) which means that AMF may produce spores at low root-colonization levels in severe saline conditions (Aliasgharzadeh et al., 2001). Landwehr et al. (2002) reported abundant occurrence of AMF spores in extremely alkaline soils of pH values up to 11, independently of the soil type and irrespective of NaCl, Na_2CO_3, Na_2SO_4 or $CaSO_4$ salt types, though the degree of colonization varied from one individual to the next.

In most of the earlier studies, identification of the AMF spores was based mainly on the morphological criteria. Complementary to morphology based identification methods, use of molecular techniques such as polymerase chain reaction and restriction fragment length polymerphism for identification of AMF has been on the rise.

There are few studies indicating that mycorrhizal fungi can increase growth of plants growing in saline habitats (Ojala et al., 1983; Pond et al., 1984). VA-mycorrhizal fungi may have the ability to protect plants from salt stress, but the mechanism is not fully understood. The few data available at present suggest that fungi do have a potential to enhance plant growth by increasing the uptake of the nutrients. Recently, Porras-Soriano et al. (2009) tested the efficacy of three species of AMF - *Glomus mosseae*, *Glomus intraradices* and *Glomus claroideum* - to alleviate salt stress in olive trees under nursery conditions. The authors observed that *G. mosseae* was the most efficient fungus in terms of olive tree performance and particularly in the protection offered against the detrimental effects of salinity. These findings suggest that the capability of AMF in protecting plants from the detrimental effects of salt stress may depend on the behaviour of each species.

Mechanisms for halotolerance

Halotolerance is the adaptation of living organisms to conditions of high salinity. High osmolarity in hypersaline conditions can be deleterious to cells since water is lost to the external medium until osmotic equilibrium is achieved. Many microorganisms respond to increase in osmolarity by accumulating osmotica in their cytosol, which protects them from cytoplasmic dehydration (Yancey et al., 1982). As biological membranes are permeable to water, all microorganisms have to keep their cytoplasm at least isoosmotic with their environment to prevent loss of cellular water; when a turgor pressure is to be maintained, the cytoplasm should even be slightly hyperosmotic. Adaptation to conditions of high salinity has an evolutionary significance. The concentration of brines during prebiotic evolution suggests haloadaptation at earliest evolutionary times (Dundas, 1998). Osmophily is related to the osmotic aspects of life at high salt concentrations, especially turgor pressure, cellular dehydration and desiccation. Halophily refers to the ionic requirements for life at high salt concentrations.

Halophilic microorganisms usually adopt either of the two strategies of survival in saline environments: ‘compatible solute’ strategy and ‘salt-in’ strategy (Ventosa et al., 1998). When an isoosmotic balance with the medium is achieved, cell volume is maintained. Compatible solute strategy is employed by the majority of moderately halophilic and halotolerant bacteria, some yeasts, algae and fungi. In this strategy, cells maintain low concentrations of salt in their cytoplasm by balancing osmotic potential through the synthesis or uptake of organic compatible solutes and exclusion of salts from cytoplasm as much as possible. The compatible solutes or osmolytes, small organic molecules that are soluble in water to molar concentrations, which accumulate in halophiles are available in great spectrum and used in all three domains of life. These are assigned in two classes of chemicals: (1) the amino acids and their derivatives, such as glycine betaine, glutamine, glutamate, proline, ectoine or N-acetyl-β-lysine and (2) polyols for example, glycine betaine, ectoine, sucrose, trehalose and glycerol, which do not disrupt metabolic processes and have no net charge at physiological pH. The accumulation can be accomplished either by uptake from the medium or by *de novo* synthesis (Shivanand and Mugeraya, 2011).

The salt-in strategy is employed by true halophiles, including halophilic archaea and extremely halophilic bacteria. These microorganisms are adapted to high salt concentrations and cannot survive when the salinity of the medium is lowered. They generally do not synthesize organic solutes to maintain the osmotic equilibrium. In this adaptation, the intracellular K^+ concentration is generally higher than that of outside, the intracellular Na^+ concentration is generally lower than that in the medium, the intracellular K^+ concentration increases with increasing external NaCl concentration in a non-linear pattern. All halophilic microorganisms contain potent transport mechanisms, generally based on Na^+ /H^+ antiporters (Oren, 1999).

Halobacillus is the first chloride-dependent bacterium reported, and several cellular functions depend on Cl^- for maximal activities, the most important being the activation of solute accumulation. *Halobacillus* switches its osmolyte strategy with the salinity in its environment by the production of different compatible solutes. Glutamate and glutamine dominate at intermediate salinities, and proline and ectoine dominate at high salinities. Chloride stimulates expression of the glutamine synthetase and activates the enzyme. The product glutamate then turns on the biosynthesis of proline by inducing the expression of the proline biosynthetic genes (Saum et al., 2012). *Halobacillus dabanensis* is used by in Beijing, China as a model organism to study the genes involved in halotolerance, including genes encoding Na^+/H^+ antiporters, enzymes involved in osmotic solute metabolism and stress proteins (Yang et al., 2006).

Applications of halophilic bacteria

Halophilic bacteria provide a high potential for biotechnological applications for at least two reasons: (1) their activities in natural environments with regard to their participation in biogeochemical processes of C, N, S, and P, the formation and dissolution of carbonates, the immobilization of phosphate, and the production of growth factors and nutrients (Rodriguez-Valera, 1993), and (2) their

nutritional requirements are simple. The majority can use a large range of compounds as their sole carbon and energy source. Most of them can grow at high salt concentrations, minimizing the risk of contamination. Moreover, several genetic tools developed for the nonhalophilic bacteria can be applied to the halophiles, and hence their genetic manipulation seems feasible (Ventosa et al., 1998).

Halophilic bacteria have the ability to produce compatible solutes, which are useful for the biotechnological production of the osmolytes. Some compatible solutes, especially glycine, betaines, and ectoines, may be used as stress protectants against high salinity, thermal denaturation, desiccation and freezing as well as stabilizers of enzymes, nucleic acids, membranes and whole cells. The industrial applications of these compounds in enzyme technology are most promising. The other compatible solutes such as trehalose, glycerol, proline, ectoines, sugars and hydroxyectoine from halophilic bacteria showed the highest efficiency of protection of lactate dehydrogenase against freeze-thaw treatment and heat stress.

Also, halophilic bacteria produce a number of extra- and intra-cellular enzymes and antimicrobial compounds that are currently of commercial interest (Kamekura and Seno, 1990). Halophilic bacteria can produce enzymes that have optimal activity at high salinity, which is advantageous for harsh industrial processes.

The application of halophilic bacteria in environmental biotechnology is possible for (1) recovery of saline soil, (2) decontamination of saline or alkaline industrial wastewater, and (3) the degradation of toxic compounds in hypersaline environments.

The use of halophilic bacteria in the recovery of saline soils is based on the hypothesis that microbial activities in saline soil may favor the growth of plants under salt stress. The second hypothesis is based on the utilization of these bacteria as bio-indicators in saline wells. Indicator microorganisms can be selected by their abilities to grow at different salt concentrations.

These organisms could indicated that well water could be used for producing low saline contamination of plants or soils which could be alleviated desertification of soil (Ramos- Cormenzana, 1993). Last hypothesis is the application of halophilic bacterium genes using a genetic manipulation technique to assist wild type plants to adapt to grow in saline soil by giving them the genes for crucial enzymes that are taken from halophiles.

CONCLUSIONS

Several studies have increased our current knowledge on different aspects of halophilic microorganisms from salted soils, such as their physiology, ecology, taxonomy or phylogenetic relationships with other microorganisms. Few hypersaline environments have been carefully surveyed using molecular methods. The recent finding on bacterial and archaeal metabolic activities suggests that these environments may harbour diverse consortia of microbes that are not easily cultured. The extremes of hypersaline environments, may yield especially interesting species. There are a number of challenges involved in describing microbial dynamics in saline soil habitats that remain unsolved. There are so many literatures that provide the basic information for the study of halophilic bacteria in saline soil including the methodology for studying halophilic bacterial density and diversity.

Aforementioned studies show that flood tolerant plants and VA mycorrhizal endobiont fungi exist together and these fungi may play role in alleviating plant stress brought caused by flooding under aquatic environments. Some recent studies suggest that VA mycorrhizal endobiont would benefit both wetland and upland rice. But there are many biological principles such as infection strategy, nutrient uptake in hypersaline conditions that need to be explained.

Besides, there are several fields in which their industrial applications are more promising, and, as in the case of other extremophilic micro-organisms, halophiles have an important biotechnological potential as a source of compatible solutes, enzymes and other compounds of industrial interest.

Moreover, moderately halophilic bacteria constitute an excellent model for the molecular study of the osmoregulatory mechanisms that permit them to grow over a wide range of salt concentrations. This aspect has very exciting potentialities. The genes involved in synthesis and accumulation of compatible solutes and their regulation have become the focus of recent investigations, for example, their possible application in agriculture to construct salt-resistant plants carrying prokaryotic genes encoding enzymes for the synthesis of osmoprotective compounds.

It is thus possible that in the future the biotechnological application of halophiles, or of genes derived from them, will extend to many more members of this extremely diverse group of microbes. Possible areas of exploitation may stretch from production of valuable compounds and remediation of contaminated waters and soils to future solutions of the world's problems.

Halophiles are likely to provide significant opportunities for biotechnology. As a result of natural and man-made global changes, hypersaline environments are on the increase. Moreover, hypersaline environments may easily be created by the concentration of sea water in arid environments. These facts, together with the occurrence of novel and stable biomolecules in halophiles, suggest that these organisms will prove even more valuable in the future.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Profile of enzymes secreted by *Nocardia* sp. isolated from sugarcane bagasse leachate and its application in degradative processes of organic matter

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Microorganisms are involved in various processes of nutrient cycling, decomposing the organic matter, producing antibiotics, biosurfactants, enzymes for various industrial applications, and may have a direct application in bioremediation strategies. At the beginning of sugarcane crushing season of 2006/2007, samples of leachate of sugarcane bagasse (which was accumulated since the 2005/2006 season) were collected in a sugar-ethanol industry from the State of Alagoas (Brazil), for the isolation of microorganisms present in the same. Among the isolated microorganisms, emphasizes was on the one encoded PB4, a bacterium of the genus *Nocardia*, due to its broad spectrum of extracellular enzymatic activities *in vitro*. Its ability to degrade cellulose in a liquid medium containing either pure carboxymethylcellulase or bagasse was also monitored, as well as its ability to degrade phenol in a liquid medium containing crushed bagasse. The cellulolytic activity was more active in media containing pure cellulose under aerobic conditions. This isolate was also more efficient to degrade polyphenols from the bagasse to simple phenols in aerobic conditions.

Key words: Actinomyces, ligninolytic activity, phenol oxidase, pulp.

INTRODUCTION

Many substances are discharged into the environment, mainly by industries, and most of these molecules are either xenobiotic/recalcitrant, or may accumulate and become even more toxic, causing serious problems. Presumably due to the fact that several of these compounds are new, most microorganisms typical of certain niches do not have appropriate metabolic pathways for their degradation.

Both natural and anthropogenic activities result in accumulation of wide ranges of toxic xenobiotic compounds in the environment, and thus cause a global concern (Gienfrada and Rao, 2008). Although some procedures

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Abbreviations: NB, Nutrient broth; **SB,** Sabouraud broth; **CMC,** carboxymethyl cellulose; **NA,** nutrient agar.

for the removal or destruction of pollutants are expensive, organic methods tend to be less costly (Alexander, 1999). Some strategies have been explored to assist the removal or degradation of pollutants in the environment. Many bacteria, fungi and certain plants have the potential to offer low-cost alternatives for its restoration. These organisms in general use the pollutants as nutrients, degrading them to benign compounds or less environmentally damaging (Bruins et al., 2000).

Microorganisms are nature's original recyclers, converting toxic organic compounds to harmless products, often carbon dioxide and water. Ever since it was discovered that microbes have the ability to transform and/or degrade xenobiotics, scientists have been exploring the microbial diversity, particularly of contaminated areas in search for organisms that can degrade a wide range of pollutants (Jain et al., 2005).

Several species of *Nocardia, Pseudomonas*, *Alcaligenes*, *Arthrobacter* and *Streptomyces* degrade simple aromatic compounds. These bacteria often act in almost anaerobic environments, consuming mainly intermediate metabolites of low molecular weight produced by fungi (Tuomela et al., 2000). The mechanism of degradation of complex pollutants involves the action of enzymes such as cellulases and ligninases, which act on recalcitrant compounds, including aromatic structure, making them accessible for assimilation (Rodrigues, 2006; Santos et al., 2008).

The sugarcane industry produces a large amount of liquid and solid wastes which, if not treated properly, have a harmful effect on the environment. Is very important to have knowledge on the enzymatic profile of microorganisms, because it allows the development of studies for production and purification with applicability in pharmaceutical, textile, leather, paper, minerals and food industries, or even the use of own microorganisms in industrial and environmental processes. The purpose of this study was to examine the biochemical and enzymatic profile of a microorganism isolated from the leachate-residue of bagasse, which was accumulated since the 2005/06 crop season of a sugar-ethanol agro-industry from the State of Alagoas (Brazil), evaluating their cellulolytic activity in liquid media containing as carbon source, pure carboxymethyl cellulose (CMC) or sugar cane bagasse *in natura*. The main advantage of the isolation of microorganisms in this environment is its ability to degrade selectivity of such compound.

MATERIALS AND METHODS

Isolation, identification and biochemical characterization

The bacterium, previously codified as PB4, was isolated at the beginning of sugarcane crushing season of 2006/07, from samples of leachate residue of sugarcane bagasse (which was accumulated since the 2005/06 crop season in the lateral area of the main unity of the "S.A. Usina Coruripe Açúcar e Alcool" (Coruripe-AL, Brazil).

Samples of leachate-residue (3 mL) were homogenized and added to nutrient (NB) and Sabouraud (SB) broths (47 mL). The cultures were subjected to agitation (120 rpm) in orbital shaker-water bath for 2 h at 40 ± 1°C. After that, aliquots of the cultures were collected and diluted in sterile water ($1:10^4$ - $1:10^1$ v/v) and 100 µL of the dilutions were inoculated in agar- sugar cane juice (25%) in Petri dishes and incubated at 30 ± 1°C (dark).

At different time intervals after incubation, the increase of different colony forming units (CFU) were observed, and they were isolated through individual replanted in new plates containing the nutrient agar medium (NA). Subsequently, a microscopic characteri-zation of the isolate through different stains (Gram, green malachite, methylene blue and fuchsine Ziehl-Nielsen) according to Neder (1992) and a biochemical characterization by inoculating the colonies in differential media (in tubes and Petri dishes) were performed as described by Vermelho et al. (2006).

The isolate was inoculated in the center of Petri dishes containing specific substrates for the evaluation of the following enzymatic activities: phenolytic (Conceição et al., 2005), ligninolytic (Dhouib et al., 2005), xylanolytic (Ferreira et al., 2002), proteolytic pectinolytic and lipolytic (Hankin and Anagostakis, 1975), carboximetilcellulolytic (Jang and Chen, 2003), amylolytic (Kiran et al., 2005) and ureolytic (Silva, 1996). After 24-96 h of incubation at 30 ± 1°C (dark), the measurements of the diameter of the halo of medium discoloration around and under the colonies were obtained. Also, tests were performed to evaluate the production of catalase and oxidase (Silva, 1996).

Submerged culture for the production of cellulase

The culture medium used was that described by Jang and Chen (2003). So, in 1 L of distilled water was suspended: 10.0 g carboxymethyl cellulose (CMC), 1.0 g peptone, 2.0 g of $(NH_4)_2SO_4$; 2.0 g of KH_2PO_4; 1.0 g urea; 0.5 g $MgSO_4.7H_2O$; 0.4 g of $CaCl_2.H_2O$ and 1 mL of trace elements solution [g/L: $FeSO_4.7H_2O$, 5.0; $MnSO_4.H_2O$, 1.6, $ZnSO_4$, 2.0; $CoCl_2$, 2.0]. The flasks (500 mL) containing 250 mL medium were inoculated with an aqueous suspension of the isolate PB4 ($1x10^6$ cells/mL; cultures 48 h old). Cultures were incubated for 144 h (30 ± 1°C, in the dark) in order to evaluate their growth under aerated (150 rpm in orbital shaker-water bath) and non-aerated conditions.

At intervals of 24 h, aliquots of 25 mL of this culture were collected and from this volume, 100 µL were removed for counting cells in a Neubauer chamber. The remaining sample was centrifuged at 5000 g (20 min, 10°C), and the supernatant was used as "crude enzymatic solution". The samples were preserved under refrigeration (6-8°C) for subsequent biochemical analysis.

Semi-solid culture for the production of cellulase

The fermentation in a semi-solid medium took place in Erlenmeyer flasks (500 mL) containing sterile culture medium (120°C, 1 atm, 20 min), which consisted of 10 g of sugarcane bagasse (previously washed in sterile distilled water, and dried in oven at 40°C for 24 h) in 200 mL of distilled water, and 1% trace elements solution (as previously described). Then, the same procedure for inoculation and counting of cells adopted in submerged fermentation was carried out.

Content of total reducing glycids

The determination of reducing carbohydrates was carried out using

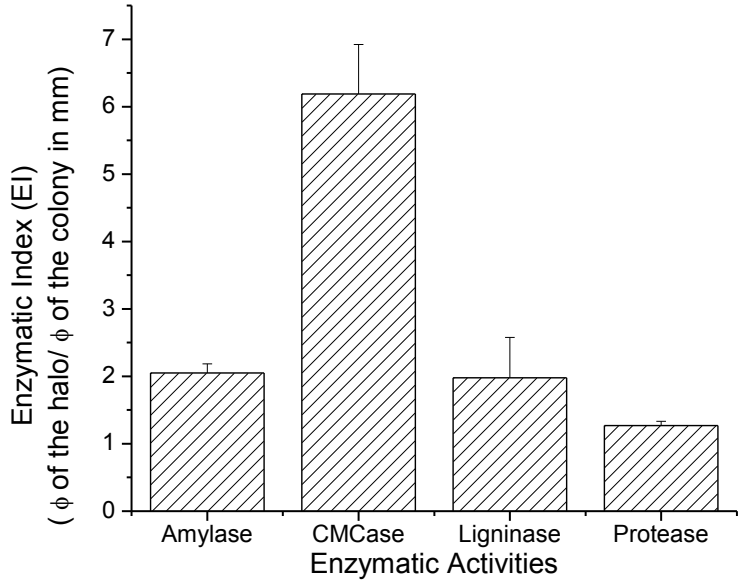

Figure 1. Main extracellular enzymatic activities expressed in solid medium by the isolate *Nocardia* sp. PB4 isolated from the leachate-residue of accumulated bagasse produced in the "SA. Usina Coruripe Açúcar e Álcool", Alagoas-Brazil (2005/06 season).

3.5-dinitrosalicilic acid (DNSA) reagent, as described by Miller (1959). The reaction mixture containing 200 µL of sample (supernatant or filtered growth medium) and 200 µL of DNSA reagent [5 g of DNSA, 100 mL NaOH (2N), 150 g $KNaC_4H_4O_6 \cdot 4H_2O$, 500 mL H_2O], was heated at 100°C for 10 min. Then, it was diluted with 5 mL of distilled water, and its absorption at 570 nm was determined in a spectrophotometer (FEMTO, XI 800). The standard curve was produced with a range of 0.2-2.0 mg/mL D-glucose (Sigma, St. Louis, MO, USA).

Content of total protein

The concentration of total protein in the samples was determined using the method described by Bradford (1976). For this, 100 µL of each sample (supernatant or filtered growth medium) was added to 250 µL of Bradford reagent (Coomassie brilliant blue G-250, Biorad®) and after 10 min, the absorbance of the reaction mixtures was measured at 700 nm in a spectrophotometer (FEMTO, XI 800). The same reaction conditions were carried out using bovine serum albumin (BSA, Gibco BRL®) as standard for the construction of the calibration curve (0-50 mg/mL).

Content of total phenols

The reaction mixture consisted of 150 µL of sample (supernatant or filtered growth medium) added, under agitation, to a volume of 3 mL of aqueous 2% Na_2CO_3. After homogenization, a volume of 25 µL of diluted (1:1 v: v) Folin Ciocalteu 2N was also added to this mixture under constant agitation (Folin and Ciocalteu, 1927). The absorbance of the reaction mixtures at 750 nm was measured in a spectrophotometer (FEMTO, XI 800) after 30 min of rest, according to Bray and Thorpe (1951). The standard curve was carried out using different concentrations of gallic acid in distilled water (0-500 µg/L) following the same reactions.

Enzymatic assay

The cellulase activity was determined by incubation of 1 mL of a crude enzyme solution (supernatant or filtered growth medium) with 1 mL of 1% CMC [substrate dissolved in phosphate buffer (100 mM, pH 7.0) at 50°C], for 60 min in water bath at 50 °C (Ghose, 1987). One unit of the enzyme activity was defined as the amount of enzyme able to release 1 µmol of glucose per minute per mL of enzyme in the reaction conditions, using a standard curve of glucose (Jang and Chen, 2003).

RESULTS AND DISCUSSION

From the 15 microorganisms isolated in leachate from sugarcane bagasse, the isolate PB4 has better performance on the production of extracellular enzymes in solid medium. It was identified as a bacterium belonging to the genus *Nocardia* (Gram positive with branched filamentous forms and weak acid-resistance), which has more than 50 species very well characterized by phenotypic and molecular methods (Trabulsi et al., 1999). They are widely distributed in the environment, particularly in soil, where they can be responsible for its characteristic odor (Conville and Witebsky, 2004).

The enzymatic tests on solid medium (Figure 1) showed positive reactions for amylase, ligninase, protease and specially cellulase, the last one produced in a higher intensity.

The occurrence of amylases in actinomycetes is a feature commonly observed in *Nocardia* and *Streptomyces* and several studies also have shown that *Nocardia* species

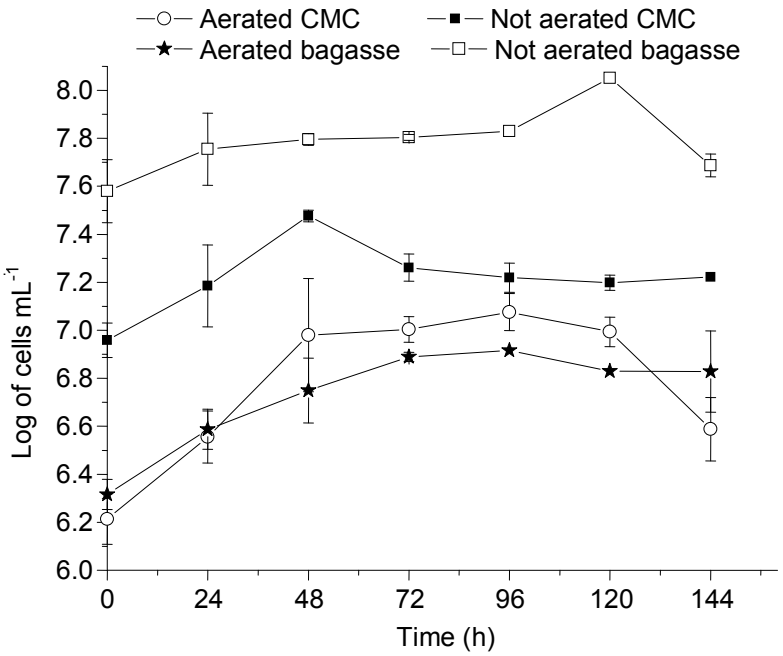

Figure 2. Growth of *Nocardia* sp. (log no. cells.mL^{-1}) isolated from the leachate-residue of accumulated bagasse produced in the "SA. Usina Coruripe Açúcar e Álcool", Alagoas-Brazil (2005/06 season), in a liquid medium containing: a) carboxymethylcellulose (CMC); b) bagasse, under conditions of aeration and without aeration (30 ± 1°C, dark).

are efficient producers of lipases (Kim, et al., 2000; Li and Rosazza, 2000), as well as some antibiotics and biosurfactants, with regards to the researches on its diversity (Kiran et al., 2005; Neder, 1992). Li and Rosazza (2000), using complete cells or enzyme preparations of *Nocardia* sp. NRRL 5646, noted that both reduced vanillic acid and the benzyl vanillic acid to vanillin by the enzyme carboxylic acid reductase that is dependent on ATP and NADPH.

The isolation of *Nocardia* sp. and *Pseudomonas* spp. in environments contaminated with oil is common, showing that this group has an important role in the degradation of hydrocarbons (Kalme et al., 2008) and very prominent with regards to its applicability in bioremediation processes. The studied bacterial strain of *Nocardia* sp demonstrated high abilities to degrade the analyzed constituents of jet fuel. No significant difference were observed between the C : N ratios (Gomes et al., 2009).

Ali et al. (2012) reported that the microorganism with the highest hydrocarbon-consumption rates in the presence of the highest heavy metal concentrations (40-80 mM sodium arsenate and 0.6-1.2 mM cadmium sulfate) was *Nocardia corallina*. In general, the filamentous bacteria seemed to be more effective in oil consumption than the unicellular bacteria in the presence of the highest cadmium sulfate concentration tested, namely 1.2 mM.

Baek et al. (2004) reported that the addition of *Nocardia* sp. H17-1 in soil contaminated with crude oil increased its degradation more efficiently than did the native microorganisms, and bioremediation with this strain has provided a reduction in the phytotoxicity of the oil in the soil to plants. Kalme et al. (2008) demonstrated the capacity of *Nocardia hydrocarbonoxydans* strain NCIM 2386 (Nh2386) to degrade diesel and kerosene with the use of Triton X-100, which increased the degradation process by reducing the time required for maximum utilization of petroleum hydrocarbons. Regarding the profile of growth of *Nocardia* sp. isolate, when grown in liquid medium containing carboxymethyl cellulose or bagasse fresh (30 ± 1°C, dark), it showed the same trend in the first 48 h under aerated conditions and not aerated (Figure 2).

According to some authors, the anaerobic incubation of *Nocardia* is not appropriate, since this showed the production of certain acids and fuels such as methane and ethanol. Bonilla and Rivas (2004) found that a moderately thermophilic and acidophilic strain of *Nocardia* sp. (EP-MC3), isolated from horse manure, degraded aerobically cellulosic substrates, due to higher biomass production and reducing carbohydrates between 40-45°C, pH 5. The authors concluded that, under aerobic conditions, this organism has the potential to produce single-cell protein and bioactive compounds from cellulosic substrates. However, the growth of the isolate *Nocardia* sp. PB4 in

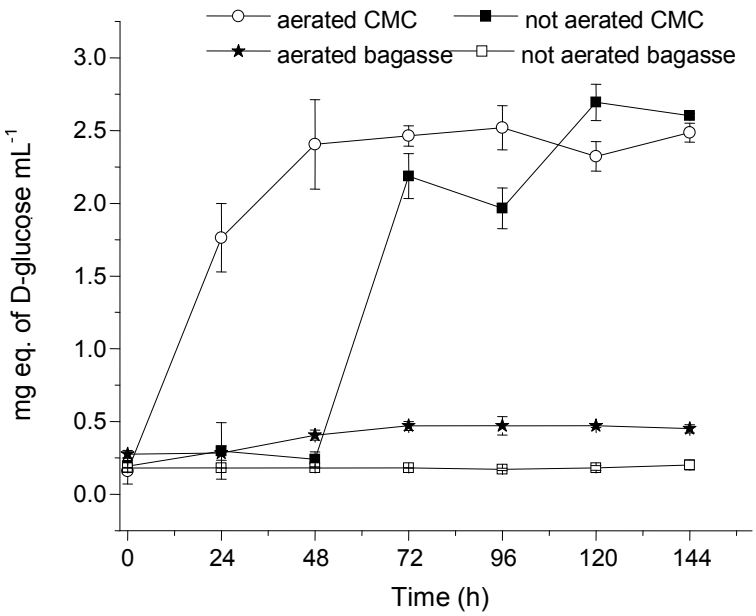

Figure 3. Time-course of the total content of reducing carbohydrates (eq. mg D-glucose. mL^{-1}) in a liquid medium containing carboxymethylcellulose (CMC) or bagasse and inoculated by *Nocardia* sp. isolated from the leachate-residue of accumulated bagasse produced in the "SA. Usina Coruripe Açúcar e Álcool", Alagoas-Brazil (2005/06 season), under conditions of aeration and without aeration (30 ± 1°C, dark).

this study was more dependent of the substrate, probably being more satisfactory under non-aerated conditions for pure carboxymethyl cellulose and bagasse.

The time-course of cellulase production (Figures 3 to 5) was evaluated by the total content of reducing carbohydrates and proteins present in the growth medium. It was observed that release of reducing carbohydrates was higher in cultures containing carboxymethyl cellulose in comparison with that containing bagasse (Figure 3), since the polymer was readily available.

On the other hand, in medium containing sugarcane bagasse (Figure 3), before accessing cellulose, the microorganism had to use other substrates, such as hemicellulose and lignin (Kewalramani et al., 1988). Induction and activity of cellulase and xylanase, vary according to the species, medium pH, availability of mineral salts and incubation time, because their products are not necessarily synchronized, leading to a *lag* phase depending on the type of substrate (Wong and Saddler, 1992).

Considering the supply of oxygen, enzymes to degrade previously lignin probablly require an aerated condition, because when sugarcane bagasse was used as substrate for the action of *Nocardia* sp, under non-aerated conditions, carbohydrates were hardly detected (Figure 3) to just 144 h of cultivation. Under aerobic conditions, these have been especially detected between 24 and 72 h (Figure 3), when the largest growth of the microorganism occurred under the same conditions (Figure 2).

Concerning the content of total protein present in the medium, not aerated cultures (Figures 4) showed peaks between 72-96 h (carboxymethylcellulose) and 120 h (bagasse). The protein content of aerated culture containing carboxymethyl cellulose remained virtually unchanged during the incubation period. In sugarcane bagasse-medium, under aerated conditions, there was intense growth and higher levels of total protein, which is explained by the requirement of other oxidative enzymes (proteins) to the degradation of this substrate.

The same is noticed when analyzing the cellulolytic activity in terms of glucose production (Figure 5). The concentration of this molecule rose throughout the period of aerobic incubation in medium containing bagasse, that is, as other oxidative enzymes degrade other polymers, cellulose became available to generate glucose. In culture containing only cellulose, even under non-aerated conditions, cellulolytic activity was more intense, as the pure substrate was accessible during the entire period (Figure 5). So, the highest growth in medium containing carboxymethyl cellulose (Figure 2) in comparison with that containing bagasse (Figure 3) reflects these differences.

Figure 6 shows the content of total phenols in equivalent mg of gallic acid in the culture medium containing bagasse, since this residue is rich in the phenolic polymer

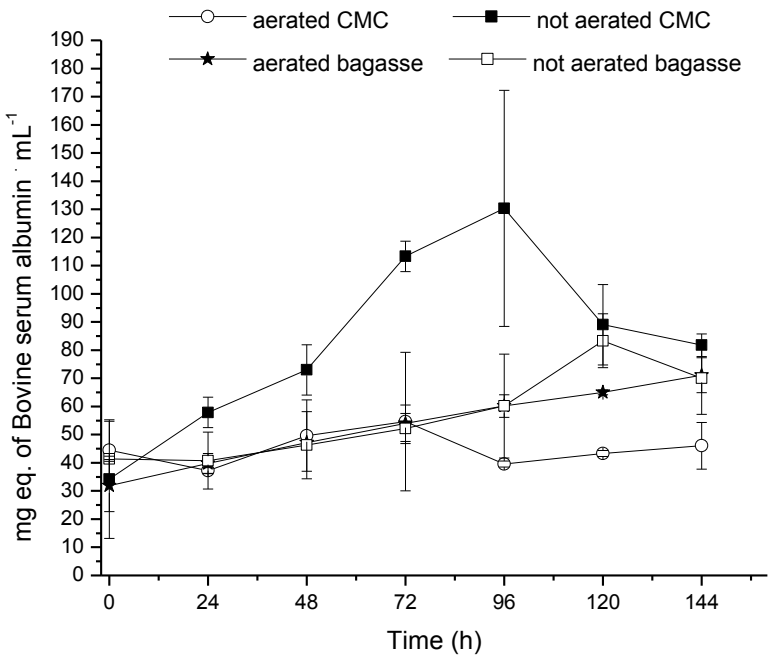

Figure 4. Time-course of the total content of proteins (eq. mg bovine serum albumin. mL^{-1}) in a liquid medium containing carboxymethyl cellulose (CMC) or bagasse and inoculated by *Nocardia* sp. isolated from the leachate-residue of accumulated bagasse produced in the "SA. Usina Coruripe Açúcar e Álcool", Alagoas-Brazil (2005/06 season), under conditions of aeration and without aeration (30 ± 1°C, dark).

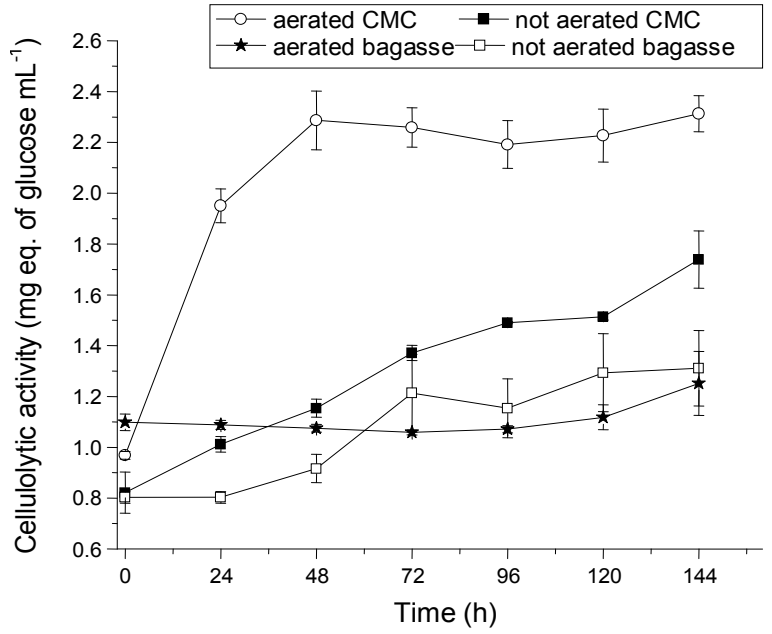

Figure 5. Time-course of the cellulolytic activity (eq. mg glucose mL^{-1}) in a liquid medium containing carboxymethyl cellulose (CMC) or bagasse and inoculated by *Nocardia* sp. isolated from the leachate-residue of accumulated bagasse produced in the "SA. Usina Coruripe Açúcar e Álcool", Alagoas-Brazil (2005/06 season), under conditions of aeration and without aeration (30 ± 1°C, dark).

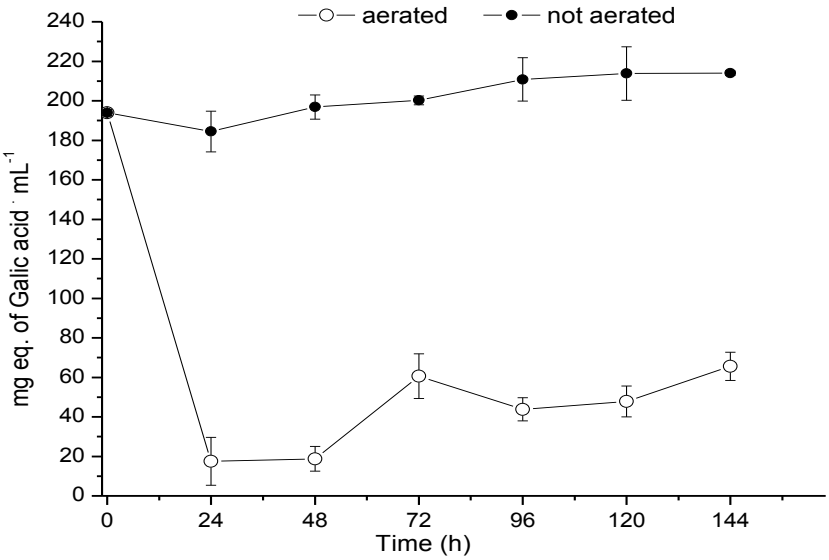

Figure 6. Time-course of the total content of phenol (eq. mg gallic acid mL^{-1}) in a liquid medium containing bagasse and inoculated by *Nocardia* sp. isolated from the leachate-residue of accumulated bagasse produced in the "SA. Usina Coruripe Açúcar e Álcool", Alagoas-Brazil (2005/06 season), under conditions of aeration and without aeration (30 ± 1°C, dark).

lignin (Kewalramani et al., 1988). It was found that there was degradation of polymeric to simple phenols, which was about 10 times higher at the first 24 h in the culture medium under aeration. This corroborates the observations that the cellulolytic activity (Figure 5) and the content of total proteins (Figure 4) were progressive in aerated condition of cultivation of *Nocardia* sp. in bagasse. According Yatome et al. (1993) *N. corallina* was able to degrade crystal violet and remove 0.49 mg/L 24 h after the inoculation, with a rate of discoloration of 0.039 mg/L/h. Species of *Nocardia, Micrococcus* and *Pseudomonas,* isolated from enriched soil/sewage sludge growth medium, were also quite efficient in the degradation of phenol and *p*-cresol in waste water seconds (Pandya, 2007). The *Nocardia* sp. strain C-14-1 isolated from the acrylic fiber wastewater could well degrade phenol while it also had high strength alkanes and succinonitrile degrading ability. C-14-1 belonged to the *Nocardia* genus according to 16S rRNA gene analysis, and it was closely related to *Nocardia amamiensis*. One catechol 2,3-dioxygenase gene was found in its genomic DNA. The phenol degradation rate was 100.0 and 42.2% with an initial concentration of 800 and 1000 mg/L within 28h (Ma et al., 2010).

Conclusions

The broad spectrum of extracellular enzyme activities displayed by the isolated PB4, from leachate of accumulated bagasse, identified as a bacterium belonging to the genus *Nocardia* sp., justified his selection for the assessment of their potential to degrade cellulose in liquid medium containing carboxymethyl cellulose pure and sugarcane bagasse. It was found that the cellulolytic activity was more active in media containing pure cellulose under aerobic conditions. The efficiency of this isolate to degrade polymeric phenols to simple phenols, in medium containing sugarcane bagasse, was also more efficient in aerobic conditions.

Considering the several reports showing the use of *Nocardia* species in bioremediation processes, particularly those related to decontamination of environments polluted by hydrocarbons and recalcitrant compounds, it is likely that the isolate tested in this study may take a place in consortia for degradation of residues containing cellulose and lignin. Studies on pilot and field scales are currently being carried out on it. The isolated *Nocardia* sp. PB4 will be subjected to molecular identification for verification of genes involved in bioremediation processes.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effects of climate change on plant associated microbial communities and enzyme activities

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Environmental alterations leads to fluctuations in the soil microbial population and soil enzyme activities, as different weather parameters affect microbial biota and their activities in rhizospheric soil of crop. An agroclimatic study was carried out to study the effect of environmental alterations on soil microbial population and enzyme activities in rhizospheric soil of rice and wheat crop. Rice and wheat crop were grown under field conditions and under temperature gradient tunnel which was maintained at 4-5°C higher temperature than open field temperature. Soil samples were taken from rhizospheric soil of rice and wheat. Microbial population was enumerated by serial dilution spread plating technique. Statistically significant higher microbial population of total bacteria, nitrogen fixers and P-solubilizers was found in rhizospheric soil samples taken from temperature gradient tunnel as compared to soil samples taken from rice and wheat grown under field conditions. Fungal population was found to be statistically, significantly higher in soil samples taken from field conditions in case of rice but, higher in soil samples taken from temperature gradient tunnel in case of wheat crop. Activities of alkaline phosphatase and dehydrogenase were assessed by using para nitro phenl phosphate (PNPP) and tri phenyl tetrazolium chloride (TTC) as substrates, respectively. Enzymes activities were found to be significantly higher in rhizospheric soil samples taken from temperature gradient tunnel. Microbial population and enzyme activities were found over a broad range of temperature but, maximum microbial and enzyme activity was found only at and near optimum conditions.

Key words: Environmental alterations, microbial activities, rice, temperature gradient tunnel, wheat.

INTRODUCTION

From ice ages to long warming periods, Earth's climate has changed drastically, during the planet's history. Climate change is one of the major challenges in 21st century faced by agriculture. In recent years, natural and anthropogenic factors have dramatically altered the composition of the atmosphere, which ultimately may modify long-term climatic trends (Baei and Risbey, 2009). Much of the ecosystem climate change research conducted to date has focused on macroscale responses to climatic change such as changes in plant growth, plant community composition, and coarse scale soil processes, many of which may also indirectly interact with effect of

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microbial processes. So, general concern about climate change has led to growing interest in the responses of soil microbial communities and their activities to altered environmental conditions (Castro et al., 2010).

Soil microbial communities are an integral component of many ecosystem processes and are responsible for the cycling of carbon and nutrients in the ecosystem (Jackson et al., 2007). Activities of these microorganisms are regulated by various biotic and abiotic factors such as quantity and quality of litter inputs, temperature, moisture, etc. (Bardgett et al., 2008). Soil microbial populations are immersed in a framework of interactions which affect plant fitness and soil quality. The great array of root-microbe interactions results in the development of a dynamic environment known as the rhizosphere where microbial communities interact. The growth and colonization of bacteria on plant root can be influenced by several soil chemical, physical and biological factors (Harries, 1998). Gaining a detailed understanding of these microbial communities regarding their relationship to environmental factors and ecosystem function, and developing methods to accurately assess them has often proven difficult (Barns et al., 1999). The principal contributor to these difficulties is the opaque nature of the soil environment, which makes direct observation of soil communities difficult. Another reason is the high diversity of these communities (Fierer et al., 2007). Amongst the various environmental factors associated with climatic changes, temperature along with moisture content is the most important environmental factor influen-cing the various processes in soil including microbial activity and microbial growth in soil. Soil temperature greatly affects the microbial population. All microbes have a set of optimal environ-mental conditions under which their growth rate is maximal, the so-called growth optima (Pettersson, 2004). Increasing temperature can increase microbial activity, processing, and turnover, causing the microbial community to shift in favor of representatives adapted to higher temperatures and faster growth rates (Castro et al., 2010).

Measurement of the soil microbial population and size of soil microbial biomass does not indicate microbial activity. Microbial activities include activities of general soil enzymes (Amador et al., 1997). Soil microorganisms are surrounded by organic matter rich in carbon and other nutrients that are required for growth and cell maintenance. But, microbes cannot directly transport these macromolecules into the cytoplasm. Rather, they rely on the activity of array of enzymes to utilize various nutrients. The rate at which soil organic matter (SOM) is decomposed is strongly affected by temperature and moisture, and thus should be sensitive to climate change. Activities of the soil enzymes are critical to the soil functioning and for maintenance of the vast biodiversity of organisms present in the soil. Soil enzymes drive soil organic matter decomposition, nutrient transformations and therefore, considered as indicator of soil health and quality. Temperature and moisture can affect both the overall rate of enzyme production and microbial activity as well as the relative rate of production of different enzymes due to effects on enzyme efficiency, substrate availability and microbial efficiency. Thus, changes in the soil microclimate, whether they occur within hours, weeks, seasonally, or over decades in response to climate change, will affect enzyme pool sizes (Steinweg et al., 2013). The link between soil microbes and their function can be made by studying the activity of enzymes involved in the C, N and P cycling (Caldwell, 2005). Soil enzymes are suitable measure to assess the effect of changing climate because they are very sensitive and respond quickly to environmental alterations. Moreover, soil enzymes are strongly associated with microorganisms. Soil enzyme activities are believed to indicate the extent of specific soil processes and in some cases they act as indicator of soil fertility. Among all enzymes in the soil environment, dehydrogenases are one of the most important, and are used as an indicator of overall soil microbial activity, because they occur intracellularly in all living microbial cells (Yuan and Yue, 2012). Alkaline phosphatase enzyme is also considered as representative of soil microbial activity. The activities of various enzymes that degrade the principle component of detrital organic matter have been extensively studied from many prospective (Kaur, 2013).

Rice and wheat are the major cereal crops cultivated in India and their growth depends upon climate, seed type and soil conditions. These both crops are primarily grown in Punjab due to rice-wheat cropping system. Although these both crops have been extensively studied but, the fundamental role of changing climatic conditions in regulating enzyme activities and microbial population of these crops under field conditions has been examined in relatively few studies. Keeping all these points in view, this research was carried out to study the effect of altered environmental conditions on microbial population and enzyme activities in rhizospheric soil of wheat and rice grown under field conditions and under temperature gradient tunnel.

MATERIALS AND METHODS

Soil sample collection

Soil samples were collected from rhizospheric soil of rice and wheat crops grown under field conditions and under Temperature Gradient Tunnel (TGT) located at the research farm of PAU, Ludhiana. The dimensions of the TGT were 30 m length × 5 m width and the meteorological parameters at the various depths within the TGT and outside were monitored at hourly interval by automated weather station manufactured by Delta -T devices, UK. Temperature and moisture within the TGT were maintained using fans, exhaust fans and coolers. TGT does not have fixed temperature. But, it always has 3-5°C higher temperature than the open field because it entraps heat radiations thus leading to higher temperature in it. TGT works on the phenomenon of greenhouse effect.

Soil temperature and moisture data recorded at different time intervals in wheat crop is represented in Table 1. Accurate recording of these parameters in rice crop was difficult due to continuous flooding in rice crop. Soil samples were collected at different time

Table 1. Weather conditions at different time intervals within TGT and open field in wheat.

Time Interval (DAS)		Wheat	
		Soil temperature (°C)	Moisture content (%)
0	F	17.5	21.6
	TGT	20.0	25.2
60	F	13.2	15.5
	TGT	17.2	16.4
120	F	23.2	15.8
	TGT	28.5	17.9
Harvesting	F	30.8	06.5
	TGT	34.7	08.7

intervals, 0, 60, 120 days after sowing (DAS) and at harvest from rhizosphere of rice crop grown during Kharif 2012 (10 June, 2012 - mid October, 2012) to wheat crop grown during rabi 2012-13 (November, 2012 - April, 2013). The crops were raised within the TGT and in open by following the crop management practices recommended in the Package of Practices of PAU, Ludhiana. Soil samples were collected by composite sampling method. Several soil samples were randomly taken from different areas of the same field. These soil samples were mixed together to get a representative sample (Walworth, 2006). A total of 16 soil samples, 4 each from rice grown under field conditions, rice grown under TGT, wheat grown under field conditions and wheat grown under TGT were collected.

Enumeration of microbial flora

Microbial populations such as total bacteria, nitrogen fixers, P-solubilizers and fungi were enumerated on Soil Extract Agar medium (Subba Rao, 1977), Jensen's medium (Jensen, 1942), Pikovaskaya's Agar medium (Pikovskaya, 1948) and Oxytetracycline Yeast Extract Agar medium (Mossel, 1970), respectively, using serial dilution spread plate technique (Kaur, 2013). All the media were prepared and sterilized in an autoclave at 15 psi pressure and 121°C temperature for 20 min.

Soil enzyme activities assay

Soil samples were analyzed for alkaline phosphatase activity and dehydrogenase activity. Alkaline phosphatase activity of soil samples was measured using para-nitrophenyl phosphate (pNPP) as substrate (Tabatabai and Bremner, 1969). It is based on colorimetric estimation of the p-nitrophenol released by phosphatase activity when soil is incubated with buffered (pH 11) sodium p-nitrophenyl phosphate solution and toluene. The colorimetric procedure used for the estimation of p-nitrophenol is based on the fact that alkaline solution of this phenol has a yellow color whereas alkaline solution of p-nitrophenyl phosphate is colorless. The intensity of yellow color is measured at 420 nm wavelength. Dehydrogenase activity of soil samples was measured using triphenyl tetrazolium (TTC) chloride as substrate (Klein et al., 1971). Measurement of dehydrogenase activity involves determi-nation of triphenyl formazan (TPF) produced by the reduction of Triphenyl tetrazolium chloride (TTC). TTC is a yellow colored water soluble salt and possesses the property of being easily transformed into intensely colored, water insoluble, methanol soluble formazan by reduction. TPF formed is extracted with methanol. The intensity of pinkish color is measured at 480 nm wavelength.

RESULTS AND DISCUSSION

Microbial population

Soil temperature greatly affects the microbial population within the soil profile. Both rice and wheat belong to C3 plant family and thus share similar metabolic characteristics. In rice crop, maximum bacterial population (310×10^6 cfu/g soil) was found at 60 DAS in soil sample taken from TGT. In case of N_2- fixing bacteria, maximum population (243×10^4 cfu/g soil) was found in soil sample taken from TGT at 120 DAS. Maximum fungal population (40×10^2 cfu/g soil) was found at 60 DAS in soil sample taken from rice grown under field conditions. Whereas, maximum P-solubilizers population of 29×10^3 cfu/g soil was found at 60 DAS in soil sample taken from tunnel (Table 2). Microbial population such as total bacteria, nitrogen fixers and P-solubilizers was found to be less in soil samples taken from rice grown under field conditions as compared to the microbial population in soil samples taken from TGT, at different time intervals. But, fungal population was found to be significantly higher in soil samples taken from rice grown under field conditions than in rice gown under TGT as indicated by the *p*-value (4.7×10^{-5}). This might be due to high temperature in tunnel which decreases fungal population as fungi is more inhibited by higher temperature than bacteria. Similar results have been reported by Pietikainen et al. (2005). They also found that fungal populations are more negatively affected by higher temperature than bacteria. This drastic decrease in fungal population at higher temperature results in an increase in the ratio of bacterial to fungal growth rate at higher temperatures. Another reason for low fungal population in the soil samples taken from TGT is high relative humidity in rice crop. Jensen et al. (2003) also stated that fungi as a group are more adapted to low soil moisture conditions than bacteria.

In the case of wheat crop, microbial population (total bacteria, nitrogen fixers, P-solubilizers and fungi) was found to be less in soil samples taken from wheat grown under field conditions as compared to the microbial population in soil samples taken from wheat grown under

Table 2. Microbial population in rhizospheric soil of rice crop grown under field conditions and under TGT at different time intervals during Kharif 2012.

Time interval (DAS)	Soil temperature (°C)	Bacterial population (cfu g^{-1}soil×10^6)	Diazotrophic population (cfu g^{-1} soil×10^4)	Fungal population (cfu g^{-1}soil × 10^2)	P-solubilizers population (cfu g^{-1}soil × 10^3)
0	33.5 (F)	59	41	19	11
	37.2 (TGT)	97	72	07	15
p-value (< 0.05)		1.86×10^{-8}	2.22×10^{-7}	4.7×10^{-5}	1.62×10^{-2}
60	27.1 (F)	286	208	40	20
	30.9 (TGT)	310	229	36	29
p-value (< 0.05)		1.65×10^{-6}	1.41×10^{-6}	4.36×10^{-5}	4.36×10^{-5}
120	28.4 (F)	219	209	29	17
	32.3 (TGT)	243	243	17	19
p-value (< 0.05)		1.69×10^{-7}	2.03×10^{-8}	1.2×10^{-5}	3.2×10^{-3}
Harvesting	25.5 (F)	112	91	31	14
	30.4 (TGT)	129	117	13	17
p-value (< 0.05)		4.96×10^{-6}	7.51×10^{-6}	5.48×10^{-6}	1.67×10^{-2}

F refers to field; TGT refers to temperature gradient tunnel.

Table 3. Microbial population in rhizospheric soil of wheat crop grown under field conditions and under TGT at different time intervals.

Time interval (DAS)	Soil temperature (°C)	Bacterial population (cfu g^{-1}soil×10^6)	Diazotrophic population (cfu g^{-1} soil×10^4)	Fungal population (cfu g^{-1}soil×10^2)	P-solubilizers population (cfu g^{-1}soil × 10^3)
0	17.5 (F)	14	13	06	03
	20.0 (TGT)	39	30	11	09
p-value (< 0.05)	(< 0.05)	1.05×10^{-6}	5.16×10^{-6}	2.7×10^{-3}	8.0×10^{-4}
60	13.2 (F)	57	136	29	24
	17.2 (TGT)	79	167	31	27
p-value (< 0.05)	(< 0.05)	7.53×10^{-5}	1.96×10^{-6}	1.3×10^{-3}	1.3×10^{-3}
120	23.2 (F)	219	111	33	07
	28.5 (TGT)	279	143	40	12
p-value (< 0.05)		2.48×10^{-9}	3.08×10^{-8}	1.0×10^{-4}	2.0×10^{-4}
Harvesting	30.8 (F)	207	97	17	11
	34.7 (TGT)	215	113	07	08
p-value (< 0.05)		4.38×10^{-9}	8.77×10^{-6}	5.0×10^{-5}	1.4×10^{-3}

F refers to field; TGT refers to temperature gradient tunnel.

TGT, at all different time intervals. At 60 DAS, the microbial count of all the microbial populations was observed to be higher than the microbial count taken at zero day of the experiment in the case of both field and tunnel soil samples. In case of total bacteria, maximum population (279 × 10^6 cfu/g soil) was found at 120 DAS. Maximum nitrogen fixers population of 167 × 10^4 cfu/g soil was found in soil samples taken at 60 DAS which may be due to increase in release of root exudates released by the crop. Maximum fungal population (40 × 10^2 cfu/g soil) was found at 120 DAS and P-solubilizers population (27 × 10^3 cfu/g soil) was found to be maximum at 60 DAS (Table 3). Maximum population of bacteria was observed whereas, P-solubilizers were very less in number. Along with the time interval, microbial populations were also found to be significantly correlated with the soil temperature at that time interval.

Changes in soil temperature affect microbial population and their activity. Data analysis revealed that maximum microbial count was found in the rhizospheric soil samples

taken from TGT than in the soil samples taken from wheat grown under field conditions (Table 3). It might be due to the fact that the soil samples were taken from wheat in winter season. Wheat crop was cultivated from November to April in Punjab state. During this period, the temperature decreases from month of November to January and then starts increasing. The minimum temperature was observed in the month of January (13.2°C) which was not optimum for the growth of micro-organisms. So, the outside temperature was very low and unfavorable for the growth of micro-organisms. The sun's energy passed through the tunnel and heated the air and soil beneath the crop. Therefore, temperature gradient tunnel resulted in increase of soil temperature from 3 to 4°C and created a favorable microenvironment for the growth of soil micro-organisms, which helped to improve and maintain the biological and physico-chemical qualities of the soils, thereby improving the growth of microbial population.

In winter, when temperature is low, the number and activity of microorganisms falls down, and as the soils warms up in summer, they increases in number as well as activity (http://www.agriinfo.in). Therefore, more microbial population was observed in rice crop as compared to wheat crop. High microbial population (total bacteria, nitrogen fixers and P-solubilizers) in rice crop (Table 2) can be attributed to presence of high moisture content in rice crop. Melentev et al. (2000) also reported similar results. They found that the moisture content and temperature of the soil considerably affect the microbial cenosis of the soil and the rhizosphere, since these factors can essentially change the amount and the pattern of nutrient secreted by plant roots. In both crops, maximum population of bacteria was observed whereas, P-solubilizers were very less in number. In the present study, significant improvement in soil microbial counts (except fungal population in rice crop) was observed in soil samples taken from rice and wheat grown under temperature gradient tunnel.

Alkaline phosphatase activity

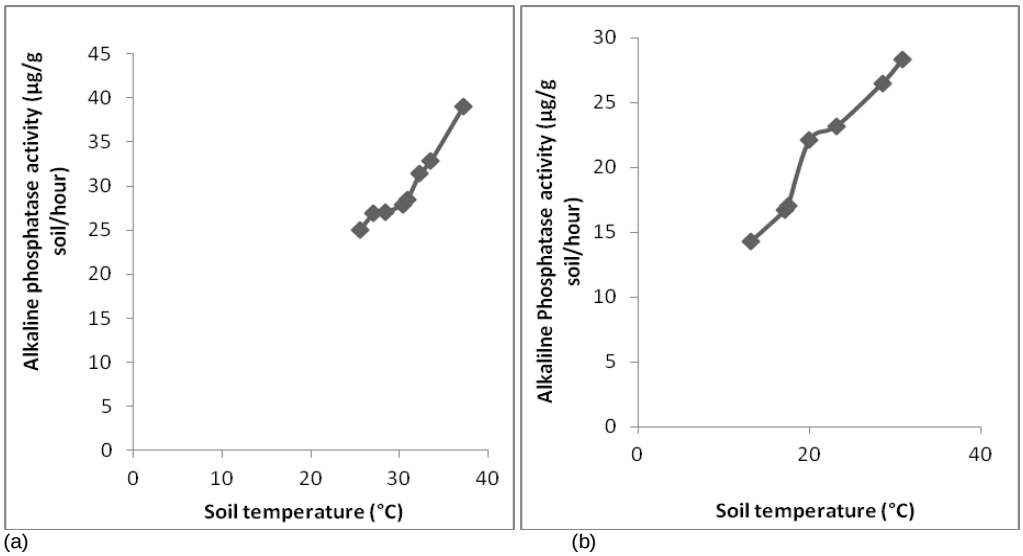
Enzyme activities of the soil are result of enzyme producing activity of soil microbes present in it. In rice crop, at zero day, alkaline phosphatase activity in rhizospheric soil samples taken from field conditions was observed to be 32.8 µg/g soil/hour at 33.5°C soil temperature. Whereas, higher alkaline phosphatase activity of 39.0 µg/g soil/hour was found in rhizospheric soil samples taken from TGT at soil temperature of 37.2°C (p-value- 2.05×10^{-10}). At 60 and 120 DAS, similar trend of significantly higher phosphatase activity in soil samples taken from TGT was observed. At the time of harvesting, alkaline phosphatase activity in rhizospheric soil samples taken from field conditions was observed to be 25.0 µg/g soil/hour at soil temperature of 25.5°C. Whereas, alkaline phosphatase activity in rhizospheric soil samples taken from TGT was found to be 27.9 µg/g soil/hour at 30.4°C soil temperature (p- value- 4.17×10^{-9}). Higher alkaline phosphates activity was found in rhizospheric soil samples taken from TGT at all the different time intervals (Figure 1a).

Similarly in wheat crop, at zero day, alkaline phosphatase activity of rhizospheric soil samples taken from field conditions was observed to be 17.0 µg/g soil/hour at 17.5°C soil temperature. Whereas, higher alkaline phosphatase activity of 22.1 µg/g soil/hour was found in rhizospheric soil samples taken from TGT at 20.0°C soil temperature (p-value- 3.49×10^{-7}). Similar trend of increased alkaline phosphatase activity in soil samples taken from TGT was observed at 60 and 120 DAS. At harvest, alkaline phosphatase activity in rhizospheric soil samples taken from field conditions was observed to be 28.3 µg/g soil/hour at 30.8°C soil temperature. Whereas, alkaline phosphatase activity in rhizospheric soil samples taken from TGT was observed to be 33.0 µg/g soil/hour at 34.7°C soil temperature (Figure 1b). Data analysis at different time intervals clearly indicated that significantly higher alkaline phosphatase was found in the rhizospheric soil of TGT than in the soil samples taken from field conditions.

An increasing trend in the alkaline phosphatase activity with increase in soil temperature was observed in both field and tunnel samples in the case of both crops because soil temperature was increasing towards the optimum temperature. The optimum temperature for soil enzyme alkaline phosphatase is 37°C. Beyond this optimum temperature, activity start decreasing with increase in temperature and at very high temperature, enzyme gets denatured. Changes in soil temperature above or below optimum temperature of enzyme would result in decrease in soil phosphatase activity. Our results are also in agreement with the study conducted by Banerjee et al. (2012). They measured p-nitro phenyl phosphate degradation over a range of temperature from 17 to 67°C and observed that degradation of p-nitro phenyl phosphate demonstrated a broad range of activity. Maximum enzyme activity was attained at 37°C. Inhibitory effects on phosphatase activity were observed above or below this temperature.

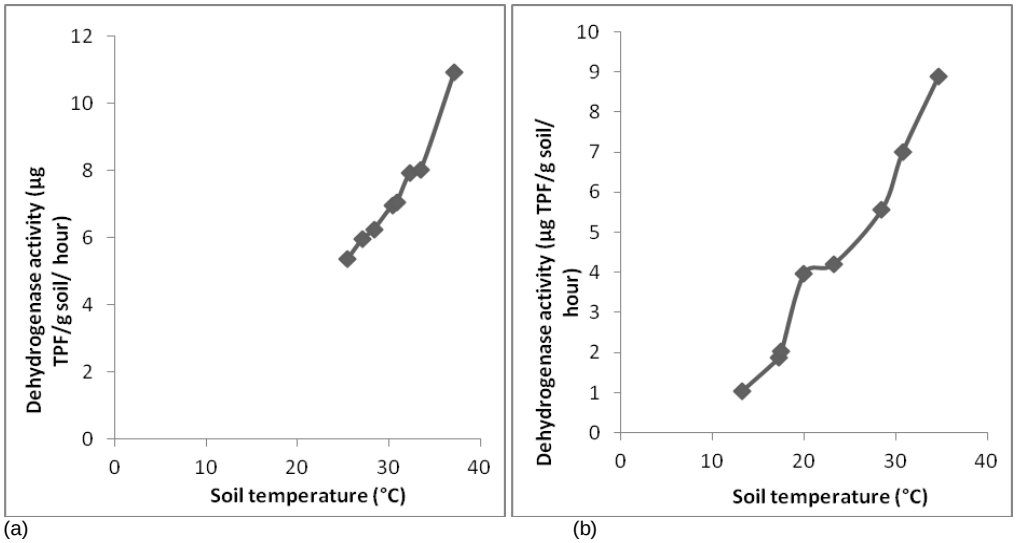
Dehydrogenase activity

Dehydrogenase enzyme activity is considered as an indicator of the oxidative metabolism in soils and thus of the microbiological activity. In rice crop, at zero day, dehydrogenase activity of rhizospheric soil samples taken from field conditions was observed to be 8.02 µg TPF/g soil/hour at 33.5°C soil temperature. Whereas, higher dehydrogenase activity of 10.94 µg TPF/g soil/hour was found in rhizospheric soil samples taken from TGT at 37.2°C soil temperature (p-value- 6.17×10^{-14}). Similar trend of significantly higher dehydrogenase activity in soil samples taken from TGT was observed at 60 and 120 DAS. At harvest, dehydrogenase activity in rhizospheric



(a) (b)

Figure 1. Variation of soil alkaline phosphatase activity with soil temperature (a) Rice crop and (b) Wheat crop.



(a) (b)

Figure 2. Variation of soil dehydrogenase activity with soil temperature (a) Rice crop and (b) Wheat crop.

soil samples taken from field conditions was observed to be 5.35 µg TPF/g soil/hour at soil temperature of 25.5°C. Whereas, dehydrogenase activity in rhizospheric soil samples taken from TGT was 6.96 µg TPF/g soil/hour at 30.4°C soil temperature. Higher dehydrogenase activity was found in rhizospheric soil samples taken from TGT at different time intervals (Figure 2a). Similarly in wheat crop, at zero day, dehydrogenase activity in rhizospheric soil samples taken from field conditions was observed to be 2.02 µg TPF/g soil/hour at 17.5°C soil temperature. Whereas, higher dehydrogenase activity of 3.97 µg TPF/g soil/hour was found in rhizospheric soil samples taken from TGT at soil temperature of 20.0°C (p-value- 4.5×10^{-13}). Similar trend of significantly higher dehydrogenase activity in soil samples taken from TGT was observed at 60 and 120 DAS. At harvest, dehydrogenase activity in rhizospheric soil samples taken from field conditions was observed to be 7.01 µg TPF/g soil/hour at 30.8°C soil temperature. Whereas, dehydrogenase activity of 8.88 µg TPF/g soil/hour was found in rhizospheric soil samples taken from TGT at soil temperature of 34.7°C (p-value- 1.0×10^{-12}). Data clearly indicated that significantly higher dehydrogenase activity was found in the soil samples taken from rhizospheric soil of TGT than in the soil samples taken from field conditions (Figure 2b).

An increasing trend in the dehydrogenase activity was

observed with increase in soil temperature in the case of both crops, because, in the present study, soil temperature were increasing towards the optimum temperature (Figure 2). So, activity of soil enzyme dehydrogenase increases significantly upto the optimum temperature beyond which activity start decreasing with increase in temperature. At a very high temperature, enzyme gets denatured.

At different time intervals, maximum dehydrogenase activity was found in the soil samples taken from TGT as compared to soil samples taken from field conditions. Results are in accordance with the study carried out by Trevors (1984) who found positive correlation between soil temperature and soil dehydrogenase activity. On comparison of enzyme activities of rice and wheat crop, it was found that activities of both enzymes were more in rice crop as compared to wheat. More enzyme activity in rice crop is also due to high moisture content in rice crop (flooding conditions in rice crop). Results are in agreement with the study conducted by Banerjee et al. (2000) and Brzezinska et al. (1998). According to them, soil water content and soil temperature influence soil dehydrogenase activity by affecting the soil oxidation-reduction status. Ross and Roberts (1970) also reported that dehydrogenase activities vary with season and are dependent on soil temperature. Present results are also supported by the study conducted be Yuan and Yue (2012). They also found lowest value of enzyme activity in winter.

Conclusion

It is concluded that soil temperature had profound effect on microbial population and enzyme activities. Though microorganisms can tolerate extreme environmental conditions, but the optimum environmental conditions at which soil microorganisms can grow and function actively is rather narrow. Soil microbial population and enzyme activities are suitable measure to assess the relationship between environment alteration and the total microbial activity but, this relationship is not always obvious, especially in the case of complex systems like soils.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Bot canker pathogens could complicate the management of *Phytophthora* black pod of cocoa

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Black pod is a major hindrance to cocoa production in Nigeria. It is caused by three different *Phytophthora* species with *Phytophthora megakarya* as the most important species in Nigeria and West African sub-region. *Phytophthora* spp. may enhance infections by opportunistic pathogens such as members of the Botryosphaeriacea that cause branch and trunk cankers in many woody plants across the world. Botryosphaeriacea has not been reported in cocoa nor in any woody plants in Nigeria to our knowledge. In the cocoa belt of Nigeria, research and understanding on cocoa black pod and *Phytophthora* is limited partly because of delayed or no access to some culture media, including required antibiotics. The objectives of this study were to: (1) use locally available materials to develop media for *Phytophthora* isolation from infected cocoa trees and pods samples and (2) to determine if members of Botryosphaeriaceae are associated with cankers of cocoa trees infected with black pod in Ondo State. The two formulated media, clarified tomato juice agar and cocoa pod agar supported the growth of *Phytophthora* spp. and were used for isolation from five cocoa producing local government areas, spanning all three senatorial districts of Ondo State. Based on morphological characteristics, four different species of Botryosphaeriaceae were identified from infected cocoa trees/pods but also from citrus and kola trees, which are similar to cocoa and usually planted in the same orchard with cocoa in Nigeria. These findings of new pathogens in cocoa and other hosts in Ondo State indicated the need for new strategies in the management of cocoa diseases in the State and across cocoa producing areas of Nigeria.

Key words: Cocoa, Canker, Black pod, pathogens, Botryosphaeriaceae, Phytophthora.

INTRODUCTION

Cocoa (*Theobroma cacao*) believed to have originated in the amazons and Orinoco basins of South America is now produced in many countries and about 70% of the world total production is from West Africa. Nigeria is the 5th largest cocoa producer in the world with 160 thousand tons which amounted to 4.6% of the world

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production in 2007 (ICCO, 2010). Cocoa is the agricultural commodity that provides the highest foreign exchange earnings in Nigeria but also supports some companies in the country. Cocoa production is concentrated in the rainforest across the 'cocoa belt' region, which include six states of Ondo, Osun, Ogun, Delta, Edo, Cross-river, and Akwa-Ibom. Ondo State is responsible for over 50% of the total national production (Adejumo, 2005; Oyekale et al., 2009).

Black pod (pod rot) is a major hindrance to cocoa production in Nigeria. It is caused mainly by three different species of *Phytophthora*: *P. palmivora, P. megakarya*, and *P. capsici* but the most important species in Nigeria and the West African subregion is *P. megakarya* (Opoku et al., 2007). Diseases caused by *Phytophthora* are very common throughout the world (Adesemoye et al., 2011), especially in wet tropical regions of the world where it cause significant crop losses such as in cocoa and palms (Opoku et al., 2007; Adejumo, 2005; Drenth and Guest, 2004; Evans et al., 2003). Except for some species that are transmitted aerially, the spread of *Phytophthora* is mainly through infected soil, water and plants and plant materials. *Phytophthora* spp. can be isolated using a selective medium consisting of V8 juice, corn meal, or potato dextrose agar as base medium and five different antibiotics, pimaricin, ampicillin, rifampicin, pentachloronitrobenzene (PCNB), and hymexazole (Kannwischer and Mitchell, 1978).

Phytophthora spp. in many pathosystems have been reported to show certain level of interaction with other pathogens. For instance, co-infecting with *Fusarium* (Adesemoye et al., 2011) and it could pave the way for other pathogens such as members of the Botryosphaeriacea (Adesemoye et al., 2014; McDonald and Eskalen, 2011). Botryosphaeriaceae are ascomycetes and rely on pruning wounds, earlier infections by other pathogens, insect infestation, and other stresses on the host for them to become opportunistic pathogens, causing branch and stem canker, gummosis, shoot blights, stem-end rot, fruit rot, and/or dieback of many woody plants (Abdollahzadeh et al., 2010; Adesemoye et al., 2013; Philips, 2002; Slippers et al., 2005).

Botryosphaeriaceae symptom expression could include dieback, bark cracking and discoloration, or death in some situations (Elliott and Edmonds, 2008; Smith et al., 1996) and cankers may exude reddish sap that dries to a whitish-beige powder. Their sporulation occurs on dead tissues and ascospores/conidia are discharged usually during wet periods. Botryosphaeriaceae possess both anamorphic (asexual stage) and teleomorphic (sexual stage) morphological characteristics. Teleomorphs of Botyrosphaeriaceae are rarely seen in nature and their morphology differ little among species but there is a wide range of differences in anamorphs morphology and are used as the basis for species identification but may be combined with phylogenetic data (Abdollahzadeh et al., 2010; Burgess et al., 2006; Luque et al., 2005). For instance, species in the genus *Diplodia*, *Dothiorella*, and *Lasiodiplodia* are separated from those in *Fusicoccum* by thick-walled conidia with a much smaller length to width ratio and are dark and septate when mature (Burgess et al., 2006; Philips et al., 2008). Phylogenetically, *Lasiodiplodia citricola* is closely related to *L. parva* but conidia of *L. citricola* are longer and wider than those of *L. parva* (Abdollahzadeh et al., 2010).

Many species of Botryosphaeriaceae have been identified in many woody plants in different parts of the world (Adesemoye et al., 2014; Brown-Rytlewski and McManus, 2000) but not in tree crops such as cocoa, citrus, or kolanut in Ondo State, Nigeria. It is common in Ondo State and many parts of Nigeria to find citrus trees planted within cocoa orchards, which make it easy for common pathogens to move between the two plants. Citrus is host to many *Phytophthora* spp. diseases such as gummosis, canker, and foot rot (Bawage et al., 2013; Drenth and Guest, 2004). Kola tree is an important woody cash crop, which is also commonly planted in cocoa orchards in Ondo State (Figure 1) and across the Nigerian cocoa belt. Though very little is known about the pathology, kola is related to cocoa and may share some common pathogens with cocoa as well as citrus. Kola tree in the genus *Cola* (Family Sterculiaceae) is native to the tropical rainforest of West Africa and is also cultivated in the American tropics (Burdock et al., 2009; www.nhm.ac.uk/seeds). The trees produce fruits, which are caffeine-containing nuts (Burdock et al., 2009). Among kola species, the most common in Nigeria are *Cola nitida* (Obi Gbanja), *Cola acuminate* (Obi Abata), *Garcina cola* (Orogbo), and *Buchholzia coriacea* ('wonderful kola'). Kola is an important economic cash crop in Nigeria, which in 2011 was reportedly worth about $30,000,000 (Akinbode, 2011). It is valued for its perceived medicinal attributes (Adebayo and Oladele, 2012; Burdock et al., 2009; Ihesie, 2013) and kola is eaten across Nigeria and used during traditional events.

The scarcity and high cost of required antibiotics and other materials for preparing growth media continue to pose a problem in many developing countries, including Nigeria (Adesemoye and Adedire, 2005) and negatively affect research on *Phytophthora* and many other pathogens. The first objective of this study was to use locally available materials to develop two media for *Phytophthora* isolation from infected cocoa trees and pods samples. The second objective was to determine if members of Botryosphaeriaceae are associated with branch and trunk cankers of cocoa trees infected with black pod in Ondo State of Nigeria and identify the species involved based on morphology.

MATERIALS AND METHODS

Sample collection

Cocoa pods showing symptoms of black pod and infected branches

Figure 1. Kola nut tree (in front) and cocoa trees (at the background) planted in close proximity and both were infected with canker but kola tree had severe mixed symptoms as shown. There was invasion by many pests on the kola tree and possibly many other opportunistic infections.

were collected from different cocoa plantation in five local government areas (LGAs) of Ondo State, Nigeria: (i) Akoko southeast, (ii) Ose, (iii) Idanre, (iv) Ondo west, and (v) Odigbo. These five LGAs spread across all the three Senatorial districts of the State that is (i) and (ii), Northern district; (iii); (iv), Central district; and (v), Southern district. Production is concentrated in the Central district. Samples showing *Botryosphaeriaceae*-like branch and/or trunk canker symptoms were aseptically collected from two other plants - citrus (sweet orange), and kolanut from two of the locations where cocoa black pod samples were collected in Oke-origbo, Akoko southeast and Ose-oba, Ose LGAs. All samples were labeled appropriately and transported to the laboratory on ice for organisms to remain viable and isolations were made from samples.

Formulation of media for isolation of *Phytophthora*

Culture media were formulated by modifying the methods of Adesemoye and Adedire (2005). Fresh and ripe tomato fruits were obtained from Ibaka market, Akungba-Akoko, Ondo State. In preparing clarified tomato juice agar (CTJA), 200 g of fresh tomato fruits without any visible infections, was weighed with a digital weighing balance (Mettler-Toledo International Inc, UK). The fruits were washed with tap water and blended. The blended chime was then sieved into a clean conical flask to remove the chaff and a volume of 100 ml of the sieved tomato chime was poured into an Erlenmeyer flask. Then, 18 g of agar-agar powder was added and sterile water was added to make a total volume of 1 L. The formulation of the cocoa pod agar (CPA) followed similar steps as CTJA. Two mature cocoa pods looking free of infection were obtained from the cocoa orchard at Oke-origbo sample location in Akungba-Akoko, Ondo State. Pods were washed, broken open, and 200 g of the pod was mashed in a mortar and the same formulation rate as CTJA was used. Each medium was homogenized, the CTJA and CPA were sterilized in the autoclave, allowed to cool to about 45°C and poured into sterile Petri dishes.

Pathogen isolation from samples

Infected cocoa pods were washed with tap water and disinfected by dropping in ethanol for 3 s, in 10% sodium hypochloride for 10 s and again in ethanol for 2 s. Due to the nature of the infection, there may be high population of saprophytes if pods are not well disinfected but the pathogen cannot be eliminated by this disinfection as they grow well into infected plant tissues. The pods were rinsed three times in sterile water to eliminate any trace of disinfectant. Aseptically, superficial tissues were removed, cuts of 2-4 mm size were made in three locations per pod using a sterile small knife and placed on the newly developed media. The inoculated plates were then incubated at room temperature (25°C) in the laboratory for 72 h. Isolates were purified by transferring hypha tips to new sterile agar plates (Adesemoye et al., 2014). Macroscopic observation of the culture was done. Microscopic examination of the sporangia was carried out by preparing slide mounts from the incubated culture plates.

Branch and trunk canker samples were wiped with serviette to remove dirt and surface-sterilized by dipping in 95% ethanol for 3 sand then passed through Bunsen flame. Samples were placed on a tray that had already been swabbed with 75% ethanol, with the aid of a small knife, each time dipping the knife into ethanol and passing it through Bunsen flame, small cut pieces of about 2-4 mm were made at the margin of healthy and infected tissues. Six pieces of each sample were placed aseptically onto a solidified potato dextrose agar with tetracycline (PDA-tet) and incubated at room temperature for 3-5 days in the dark at 25°C. Pure cultures were obtained by transferring hypha tips from the resulting colony onto fresh PDA-tet plates and incubated similarly.

For sporulation, the isolates were grown on oatmeal agar prepared as reported by Adesemoye et al. (2014) and incubated for about 3-4 weeks with light to encourage pycnidia formation. After sporulation, spores were observed under the compound microscope. Morphological examination of the conidia (spores) produced by each isolate were done, noting their colour, shape, and presence or absence of septations.

RESULTS

The two formulated media, clarified tomato juice agar (CTJA) and cocoa pod agar (CPA) both supported the growth of *Phytophthora* spp. The hyphae on the CTJA and CPA appeared whitish throughout the period of 72 h of incubation and had covered the entire length of the 100 mm Petri plate on the 4th day of incubation. With these two media, *P. megakarya* was isolated from all the five local government areas of the state where samples

Table 1. Species of Botryosphaeriaceae identified and their conidial morphology.

Isolate ID	Location of sampling	Conidial morphology	Host	Identification
OKR01	Oke-Origbo	Conidia smooth, aseptate, unicellular, cylindrical with broadly rounded ends, some with a large central guttle, smooth, with glassy wall that remains hyaline even after the conidia have been discharged from the pycnidium.	Kolanut	*Botryosphaeria stevensii*
OKR02	Oke-Origbo	Conidia oblong to subcylindrical, septate, occasionally slightly constricted at septum, moderately thick-walled, externally smooth, internally finely verruculose, ends rounded often with a truncate base.	Kolanut & cocoa	*Dothiorella viticola*
OKR03	Oke-Origbo	Dark brown unicellular Paraphyses, aseptate, thick-walled.	Cocoa	*Lasidiplodia theobromae*
OSE01	Ose-Oba	Conidial pigmented, verruculose, ovoid, 1-septate	Citrus	*Lasidioplodia citricola*
OSE02	Ose-Oba	Dark brown unicellular Paraphyses, aseptate, thick-walled.	Cocoa	*Lasidiplodia theobromae*
OSE03	Ose-Oba	Dark brown unicellular Paraphyses, aseptate, thick-walled.	Cocoa	*Lasidiplodia theobromae*

Figure 2. (A) Conidia of *Botryoshaeria stevensii* OKR01 isolated from kola tree. **(B)** Front. (**C)** Reverse view of *Botryosphaeria stevensii* on oatmeal agar at 3 weeks. Mycelium was dense and aerial. Pycnidia were visible.

were collected: Akoko southeast, Ose, Idanre, Ondo west, and Odigbo LGAs.

Characterization of isolates from branch and trunk canker samples revealed four species of *Botryosphaeriaceae* from cocoa as well as two other hosts (citrus and kola tree) obtained from two sampling locations (Akungba-Akoko and Ose) in Ondo State (Table 1). No sexual structures were observed and observations were based on anamorph structures produced by the isolates on oatmeal agar at 3 weeks. The species identified included *Lasidiplodia theobromae* and *Dothiorella viticola* isolated from cocoa, *Botryosphaeria stevensii* and *Dothiorella viticola* isolated from kolanut tree, and *Lasiodiplodia citricola* isolated from citrus, details of which are presented in Figures 2 to 5.

B. stevensii isolated from kola tree has similarities to

Figure 3. A Conidia of *Dothiorella viticola* OKR 2. **(B)** Front. (**C)** Reverse view of *Dothiorella viticola* on oatmeal agar at 3 weeks. Possesses aerial mycelium, colonies cottony, dark olive to grayish, darkening from the centre of the colony. Individual pycnidia were visible, spherical to globose, black, immersed, partially erumpent and thick-walled at maturity.

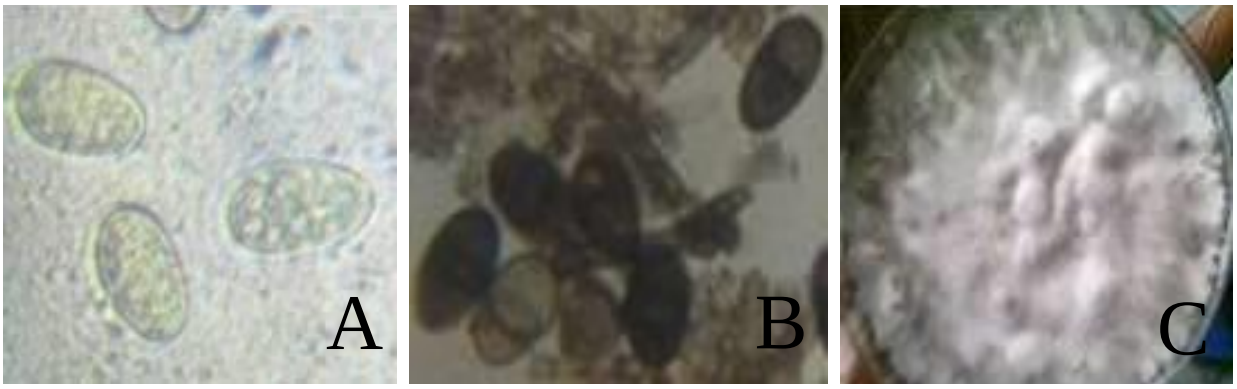

Figure 4. (A) Young conidia of *Lasidiplodia theobromae* OKR3. (**B)** Paraphyses, young and matured conidia of *Lasidiplodia theobromae* OSE2. **(C)** Front view of *Lasiodiplodia theobromae* on oatmeal agar at 3 weeks. Hyphae greyish and fluffy with abundant aerial mycelium; the reverse of the colony ranges from fuscous to dark at 4 weeks. Pycnidia were looking pronounced and raised.

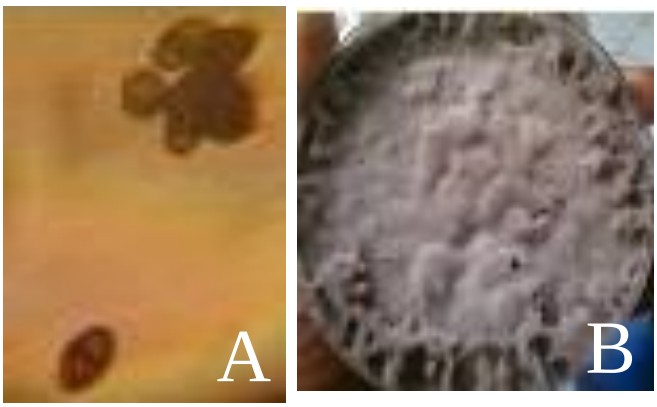

Figure 5. (A) Matured conidia of *Lasiodiplodia citricola* OSE1. (**B)** Front view of *Lasiodiplodia citricola* on oatmeal agar at 3 weeks. Presented abundant aerial mycelium reaching to the lid of the petri plate, becoming smoke-grey to iron grey at the surface and greenish grey at the reverse of the plate. Pycnidia were superficial, dark brown to black, covered with mycelium.

the characteristics described by Alves et al. (2004) and Philips (2002) with conidia that are smooth, unicellular, cylindrical with broadly rounded ends, a thick glassy wall that remains hyaline even after the conidia have been discharged from the pycnidium. It had previously been reported as the cause of branch canker in grapevine (Philips, 2002; Larignon and Dubos, 2000). *D. viticola* isolated from kola tree were similar to those described by Philips et al. (2005) and Luque et al. (2005), with brown and 1-septate conidia that darkens from early stages of development.

L. citricola isolated from citrus had similar characteristics to the description by Abdollahzadeh et al. (2010) with conidia initially hyaline, aseptate, ellipsoid to ovoid, both ends broadly rounded becoming pigmented, verruculose, ovoid, and 1-septate with longitudinal striations. On the plate, *L. theobromae* showed fluffy, irregular and cottony white appearance and later turned black similar to the description by Abdollahzadeh et al.

Figure 6. Detached cocoa pods left in the plantation that may act as sources of further infection for *Phytophthora* spp. and members of the Botryosphaeriaceae.

(2010) and the mature conidia had 2-celled dark brown conidia with striation (Burgess et al., 2006).

DISCUSSION

The developed media was helpful in the isolation of *P. megakarya* from all five sampling locations. The results indicate that *P. megakarya* is the common specie of *Phytophthora* in all the three districts of Ondo State. The problem usually encountered in Nigeria from the unavailability of several antibiotics that are added to the conventional or popular medium used in *Phytophthora* studies may be circumvented. The clarified tomato juice agar (CTJA) and cocoa pod agar (CPA) did not require any antibiotic.

The pattern of growth of the pathogen on the formulated media was different from the popular medium. On the conventional medium, *Phytophthora* growth is usually slower appearing whitish with appressed mycelia growth pattern while on the newly formulated CTJA and CPA, the mycelia growth had fluffy aerial whitish appearance and fast growth on the two media, which might be due to the absence of antibiotics on the two formulated media but growth was slightly faster on CTJA than CPA. On both media, the aerial mycelia growth first became obvious after 72 h of incubation at 25°C. Further studies for the isolation of *Phytophthora* species with these media are recommended for necessary improvements and modifications and possible commercialization.

Cocoa is an important cash crop in Nigeria and West Africa on which about one million people, mostly small-holder farmers, directly derive their livelihood (Opoku et al., 2007) and the livelihood of many more are indirectly dependent on it. The infestation of cocoa trees by members of Botryosphaeriaceae which cause dieback and their possible interaction with *Phytophthora* black pod disease constitute additional concerns to cocoa production in West Africa. New strategies for managing both diseases are urgently needed to improve cocoa production in Nigeria, where production has been decreasing. Canker disease will add more complexity to disease management in cocoa as the pathogen has now been isolated from cocoa but also from two other cash crops - citrus and kola trees in the region.

Botryosphaeriaceae are difficult to control once established. It has been shown that their spores could persist in the soil and leaf litter in the orchard and they are likely to invade plants particularly during establishment (seed and soil transmission), during stress, and shortly after pruning, as pruning wounds provide a major entry point for potential pathogens (Adesemoye et al., 2014; McDonald and Eskalen, 2011). Sanitation by total removal of infected parts and pruned branches as well as treatment of wounds on trees appear to be the best option to reduce disease. Though chemicals are used, none is labeled for the pathogen (Peterson and Helmer, 1992). Pods that are usually left within cocoa plantations (Figure 6) should be removed as this will help to reduce potential inoculum sources of *Phytophthora*, Botryosphariaceae, and other related pathogens.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

The specific nutrient synergy and their effect on the reduction of pathogens resistance to antibiotics

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The high level of pathogens resistance is becoming a huge problem in the health care system. The difficulty to treat recurrent infections also contributes to the spread of resistant microorganisms in the environment. The antibacterial therapy is very often not effective and costly. On the other hand, there is a growing interest in using natural plant components as effective antibacterial agents and valuable complement to the anti-infective therapy. The aim of this study was to determine the modulation of bacterial resistance by Epi-Quercican™, a synergistic combination of specific plant extracts, amino acids, minerals and vitamins. The Gram-positive and negative clinical strains isolated from several clinical materials, hospital equipment and environment were cultivated in the brain heart infusion broth in absence and presence of Epi-Quercican™ at 37°C for 2 h. In both cases, the sensitivity to antibiotics was tested by the disc-diffusion method. Epi-Quercican™ was efficacious against the tested pathogens and significantly reduced to the level of their resistance. The results suggest that the nutrient synergy contained in Epi-Quercican™ can be utilized in the treatment of infectious diseases.

Key words: Multidrug resistance, Epi-Quercican™, *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Enterobacter cloacae*, *Acinetobacter baumannii*.

INTRODUCTION

It has been found that after a stormy bloom of medicine related to synthetic drugs, means of natural origin are used more often. For instance most of the Brazilian population (80%) consumes only 37% of the commercially available drugs and depend almost exclusively on medicines of natural origin (Funari and Ferro, 2010). This country has been famous for the world's highest biodiversity, accounting for over 20% of the total number of known species. Each geographic region is abundant in many valuable medicinal plants which have long been

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used in folk medicine. A lot of them have been described in native pharmacopeia. However, thanks to areas such as systematic botany, phytochemistry, phytopharmacology or biotechnology, the properties of various active plant metabolites are becoming increasingly known. Drugs of natural origin are becoming more popular because it was noticed very quickly that synthetic drugs have numerous side effects which are often difficult to predict, require a complex manufacturing process and are expensive. Moreover, a huge problem with infections caused by multidrug resistant pathogens calls for the continuous search for effective antimicrobial agents. The necessity of researching new medications is largely the result of micro-organisms ability to develop different mecha-nisms of resistance to the recommended and commonly used synthetic drugs. Among them are multidrug resistant strains of staphylococci as methicillin-resistant *Staphylococcus aureus*- MRSA, methicillin-resistant *Staphylococcus epidermidis*- MRSE and other coagulase-negative staphylococci- MR-CNS, enterococci resistance to aminoglycosides (HLAR) and glycopeptide (VRE) and Gram-negative ones having the capacity to produce plasmid beta-lactamase with extended-spectrum (ESBLs) hydrolyse all penicillins antibiotics monobactams and cephalosporins, even those with a wide range of activity (DeLeo et al., 2010; Courvalin, 2005; Meyer et al., 2010; Galani et al., 2008). The complex activity of plant metabolites, as well as their synergy of action with antibiotics may contribute to the reduction of the therapeutic doses of synthetic drugs (Silva and Fernandes Júnior, 2010; Tan and Vanitha, 2004; Yang et al., 2012; Sung and Lee, 2008; da Silva et al., 2014). Zhao at al. (2001) showed that epigallocatechin gallate (EGCg) have inhibi-ting activity for *S. aureus* MRSA and MSSA strains with the MICs 100 mg/ml or less. They confirmed the synergy against MRSA and MSSA in the combinations between EGCg and all types of tested β-lactams, including benzylpenicillin, ampicillin, oxacillin, methicillin and cephalexin (Zhao et al., 2001). According to Zhi-Qing et al. (2002) epigallocatechin gallate and carbapenems showed potent synergy against MRSA. Because of the interesting research by Harakeh et al. (2013) associated with the modulation of resistance in bacteria isolated from dairy products, we decided to check the modulation of resistance by Epi-Quercican^{TM} in bacteria isolated from difficult to treat clinical case. The aim of this study was to investigate the effect of Epi-Quercican^{TM} on the pathogenic bacterial resistance reduction.

MATERIALS AND METHODS

Bacterial strains isolation and identification

The bacterial strains were from different materials taken from patients, hospital equipment and environment in the internal medicine, surgical ward, urological wards and intensive care unit of several hospitals in Lodz, Poland. Bacterial strains from *S. aureus* genera (n=20) were isolated from wounds, ulcers, bronchial secretions, abdominal cavity exudates and blood; *Enterococcus faecalis* (n=12) strains from wounds, urine, hospital equipment, and hospital environment; clinical strains of *Escherichia coli* (n=30) from wounds, bronchial secretion, abdominal cavity, blood, urine *Enterobacter cloacae* isolates (n=20) from abdominal cavity, wounds, ulcers, bronchial secretion, urine, blood, and hospital environment; and *Acinetobacter baumannii* strains (n=16) from bronchial secretion, wounds, urine, sputum, anus and hospital environment.

Staphylococci clinical strains were identified according to standard methods of culturing on Columbia Agar (bioMerieux), on Mannitol Salt Agar (bioMerieux), and determining the ability of bacteria to produce catalase and coagulase (bioMerieux) and using API Staph tests (bioMerieux). Enterococci were identified to the genus level on Columbia Agar (bioMerieux), Enterococcosel Agar (Emapol) and using API 20 Strep tests (bioMerieux). Gram-negative bacteria were cultured with use of Columbia Agar (bioMerieux) and Mac Conkey Agar (bioMerieux). They were identified to the species level with the use of API 20 E and API 20 NE tests (bioMerieux) according to manufacturer's instructions. The bacteria were incubated at 37°C for 24 h.

Epi-Quercican^{TM} is composed of the following ingredients in the relative amounts indicated: 125.8 mg of vitamin C (as ascorbic acid, Mg, Ca and palmitate ascorbate), 166.7 mg of L-lysine, 125 mg of L-proline, 83.3 mg of L-arginine, 33.3 mg of N-acetyl L-cysteine, 166.7 mg of standardized green tea extract (80% polyphenols), 8.3 mg of quercetin, 5 µg of selenium, 333 µg of copper and 167 µg of manganese.

Bacterial growth conditions

All isolates of *S. aureus*, *E. faecalis*, *E. coli*, *E. cloacae*, *A. baumannii* were grown in 5 ml brain heart infusion at 37°C for 18 h. A total of 100 µl of culture were added to 5 ml of brain heart infusion broth containing Epi-Quercican^{TM} at 25 and 50 mg/ml. Both concentrations were used for the tests of reducing microbial resistance. The mixtures were incubated in a 37°C shaker. Samples from two concentrations were taken at two different time-points of 1 and 2 h in the preliminary study. Bacterial susceptibility to the recommended antibiotics testing was conducted by disk-diffusion method before and after treatment with Epi-Quercican^{TM}. The change in the resistance pattern was calculated and expressed in percent.

Bacterial susceptibility to antibiotics

Bacterial cultures of the tested strains on Columbia Agar medium at 37°C for 24 h were performed. Inoculum and optical density of 0.5MF (bioMerieux densitometer) was applied on Mueller-Hinton II Agar (bioMerieux) and incubated at 37°C for 18 h. Susceptibility testing was carried out with the use of the disk-diffusion method with the following antibiotics (Becton Dickinson) used against all tested *S. aureus* and *E. faecalis* clinical strains: GM- gentamicin (10 µg), CIP- ciprofloxacin (5 µg), C- chloramphenicol (30 µg), TE- tetracycline (30 µg), TGC - tigecyclin (15 µg), and VA- wankomycin (30 µg); only for *S. aureus* strains: AN- amikacin (30 µg), NET- netilmicin (30 µg), TOB- tobramycin (10 µg), SXT- trimethoprim/sulfamethoxazole (1.25 µg/23.75 µg), FOX- cefoxitin (30 µg), E- erythromycin (15 µg), DA- clindamycin (2 µg), RA- rifampicin (5 µg), LZD- linezolid (30 µg), FD- fusidic acid (10 µg), QD- quinupristin/dalfopristin (15 µg), K- kanamycin (30 µg), MUP- mupiracin (200 µg); and only for *E. faecalis* strains: RA- rifampicin (5 µg), LZD- linezolid (30 µg), P- penicillin 10 IU, AM- ampicillin (10 µg), TEC- teikoplanin (30 µg), N/F - nitrofurantoin (300 µg), NOR-

norfloksacin (10 µg), FOS- fosfomycin (200 µg), S- streptomycin (300 µg). For all clinical strains of *E. coli*, *E. cloacae* and *A. baumannii* were used: GM- gentamicin (10 µg), PIP- piperacillin (100 µg), TIC- ticarcillin (75 µg), TZP- piperacillin/tazobaktam (100/10 µg), TIM- ticarcillin/clavulanic acid (75 µg /10 µg), CTX- cefotaxim (30 µg), CAZ- ceftazidime (30 µg), FEP- cefepime (30 µg), ATM- aztreonam (30 µg), IPM- imipenem (10 µg), MEM- meropenem (10 µg), ETP- ertapenem (10 µg), DOR- doripenem (10 µg), CIP- ciprofloxacin (5 µg), AN- amikacin (30 µg), NET- netilmicin (30 µg), TOB- tobramycin (10 µg), C- chloramphenicol (30 µg) and SXT- trimethoprim/sulfamethoxazole (1.25 µg /23.75 µg); for clinical strains of *E. coli* and *Enterobacter cloacae*: CXM- cefuroxime (30 µg), TE- tetracycline (30 µg), TGC- tigecyclin (15 µg), only for *E. coli* strains: AMC- amoxicillin / clavulanic acid (20 µg /10 µg), CF- cefalotin (30 µg), CZ- cefazolin (30 µg), AM- ampicillin (10 µg), FOX- cefoxitin (30 µg); and only for *A. baumannii* strains: SAM- ampicillin/sulbactam (10/10 µg) and CL - colistin (50 µg).

S. aureus ATCC 29213, *E. faecalis* Van B ATCC 51299, *E. faecalis* ATCC 29212, *E. coli* ATCC 25922 and *A. baumannii* ATCC 19606 strains were used as a control.

The results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.

RESULTS

The bacterial resistance to antibiotics

S. aureus strains isolated from wounds, ulcers, bronchial secretions, abdominal cavity exudates and blood were resistant to: E (100%); K (90%); DA (70%); CIP, AN (60%); TOB, C, FOX, MUP (40%); GM, TE, RA, LNZ (30 %); FD, QD (20%); SXT (10%); VA and TGC (0%). *E. faecalis* tested strains which were isolated from wounds, urine, hospital equipment, and hospital environment showed resistance to: GE (100%); TE (90%); S (80%); P, AM (70%); C (60%); CIP (50%); NOR (50%); TGC (40 %); RA, LNZ (30%); F/N (10%); and VA, TEC, FOS (0%). The resistance of *E. coli* isolates was as follows: AMC, AM, PIP, TIC, TE, SXT (90%); TIM (80%); C (70%); CIP, NET, CXM, AN (60%); CZ, CAZ, FEP, GM, TZP, ATM (50 %); CTX (20%); CF, IPM, TGC, TOB (10%), FOX, MEM, ETP, DOR (0%).

Enterobacter cloacae strains isolated from abdominal cavity, wounds, ulcers, bronchial secretion, urine, blood and hospital environment were resistant to: GM, TOB, CXM (90%); PIP, TIC, SXT (80%); CAZ, TE (70%); CTX, CIP (50 %); ATM, AN (40%); FEP, NET, C (30%); TZP, TIM (20%); TGC (10%); IPM, MEM, ETP, DOR (0%). Tested strains from *A. baumannii* genera were characterized as the most resistant to recommended antibiotics. As such, isolates from bronchial secretion, wounds, urine, sputum, anus, and hospital environment were 100% resistant to: PIP, TZP, CTX, CIP, TOB, C, SXT and 90% to: TIC, TIM, CAZ, FEP, AN, NET. 50% of tested strains were resistant to carbapenems: IPM, MEM, ETP and DOR. Only colistin was active against all *A. baumannii* isolates.

The reduction of tested bacterial strains resistance by Epi-Quercican™

The effect of Epi-Quercican™ on the microbial resistance profile of the tested bacterial strains was concentration dependent. Reduction of bacterial resistance was most pronounced at Epi-Quercican™ concentrations of 50 mg/ml and after 2 h of incubation. The majority of bacterial strains from *S. aureus* genera were resistant to E and K. In the presence of Epi-Quercican™, the resistance to these antibiotics was reduced from 100 to 60% and from 90 to 60%, respectively. In addition, the significant decrease of resistance to C (from 40 to 10%), RA and LNZ (from 30 to 10%) was observed. Epi-Quercican™, however, did not affect the resistance of *S. aureus* tested clinical strains to CIP. The modulation of microbial resistance of *S. aureus* isolates by Epi-Quercican™ is presented in Figure 1.

Epi-Quercican™ did not have major effect on the sensitivity improvement of *E. faecalis* clinical isolates to GM (change from 100 to 90%) and similarly to TE, S, C with the reduction of resistance by about 20%. After exposure to Epi-Quercican™ a larger number of enterococci isolates displayed higher sensitivity to TGC, RA and LNZ. No change in the level of resistance to P and AM was observed. The modulation of microbial resistance of *E. faecalis* isolates by Epi-Quercican™ is presented in Figure 2. The greatest effect of resistance modulation by Epi-Quercican™ in *E. coli* clinical strains was seen against TIC, TE, CIP, NET, CXM, GM, TZP, ATM resulting in lowering the resistance to these antibiotics by about 20%. There was no increase in sensitivity against PIP and SXT. The modulation of microbial resistance of *E. coli* isolates by Epi-Quercican™ is presented in Figure 3. *E. cloacae* clinical isolates exposed to Epi-Quercican™ become more sensitive to CAZ with the reduction of resistance by about 30% and to CXM, TE (reduction by about 20%). For these genera of bacteria the sensitivity to other antibiotics, such as GM, SXT, CIP, AN and C was unaffected by Epi-Quercican™. The modulation of microbial resistance of *E. cloacae* isolates by Epi-Quercican™ is presented in Figure 4.The modulating properties of Epi-Quercican™ in reducing the level of antibiotic resistance of *A. baumannii* strains were mainly seen for PIP, TZP, CTX, TOB, SXT and ATM. In this case, the resistance to antibiotics was decreased by about 20%. The resistance to antibiotics: CIP, C, CAZ, FEP and AN was not affected.

The modulation of microbial resistance of *A. baumannii* isolates by Epi-Quercican™ is presented in Figure 5.

DISCUSSION

In general, the sensitivity of all tested clinical strains to most recommended antibiotics has significantly improved in the presence of Epi-Quercican™. The results of

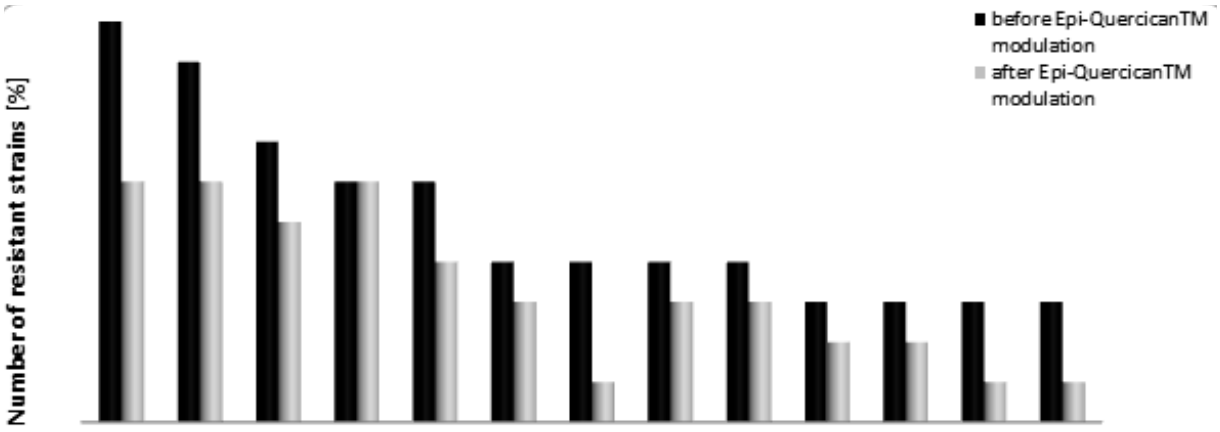

Figure 1. Modulation of microbial resistance of *S. aureus* isolates by Epi-Quercican^{TM}.

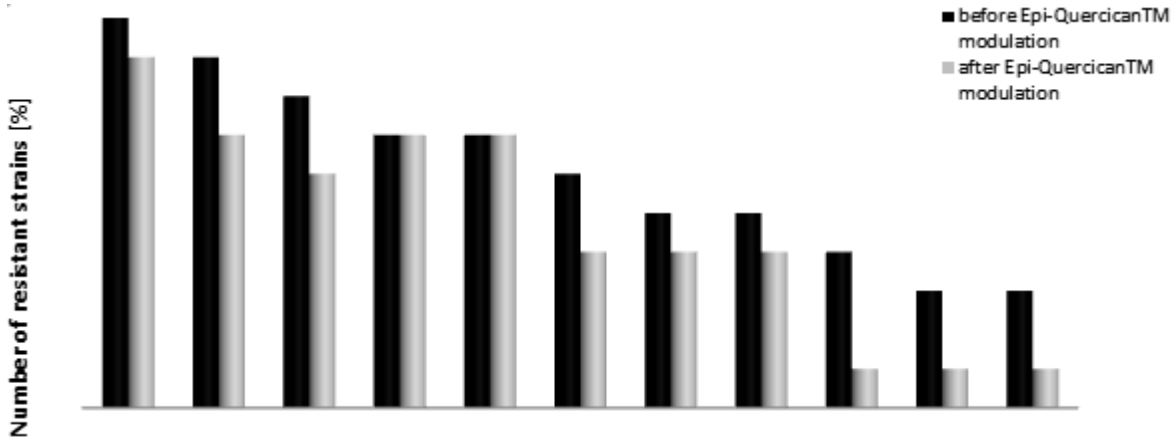

Figure 2. modulation of microbial resistance of *E. faecalis* isolates by Epi-Quercican^{TM}

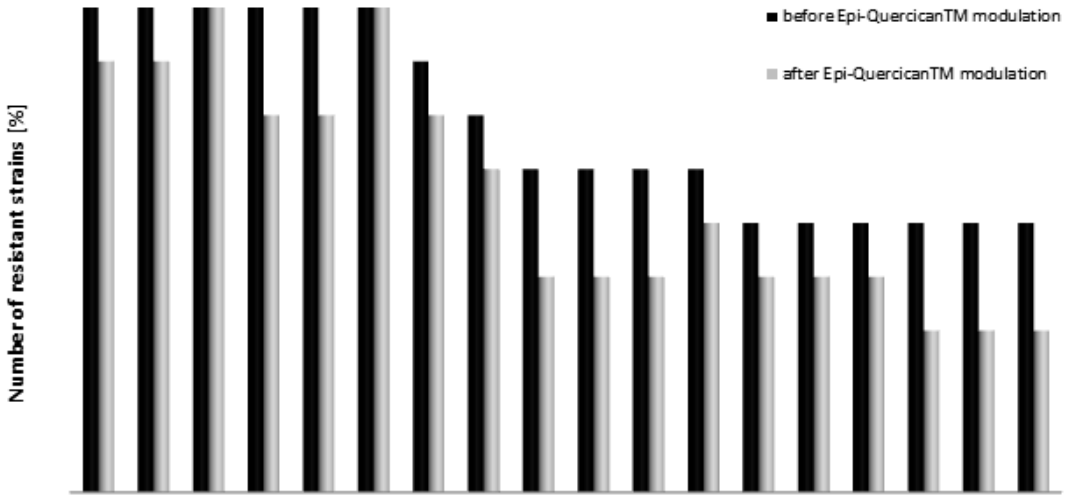

Figure 3. Modulation of microbial resistance of *E. coli* isolates by Epi-Quercican^{TM}

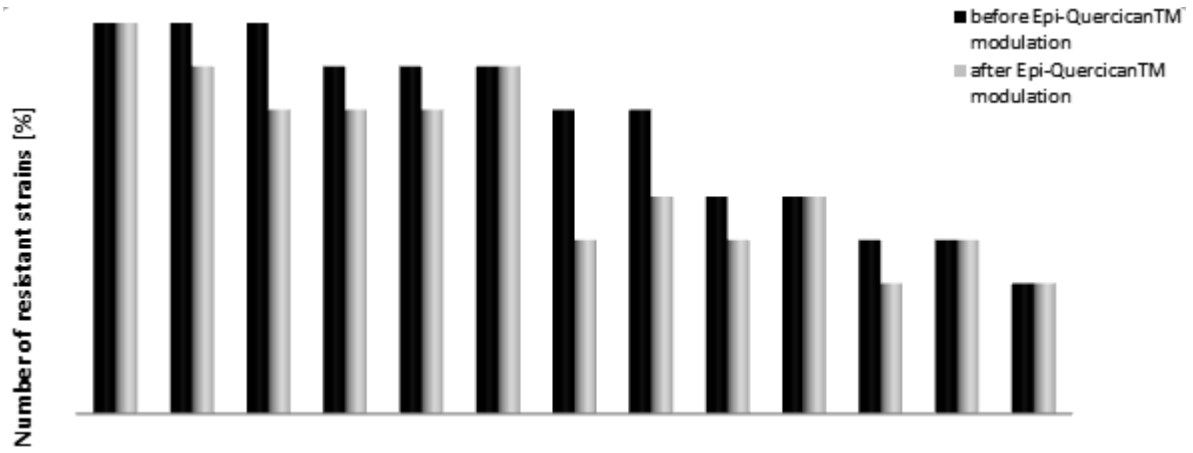

Figure 4. Modulation of microbial resistance of *E. cloacae* isolates by Epi-Quercican™

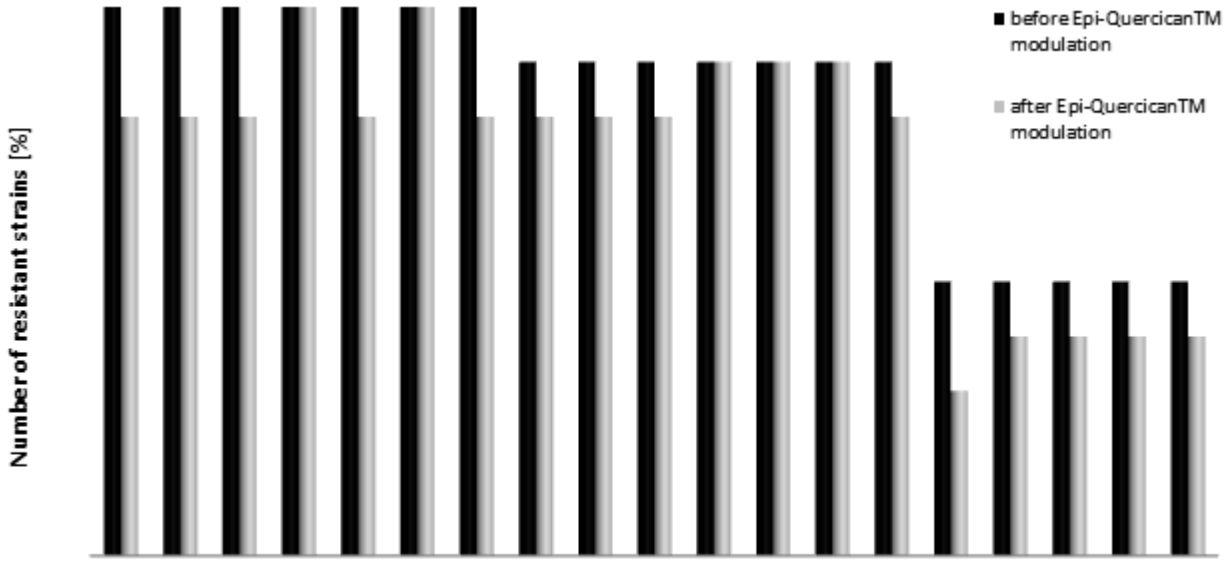

Figure 5. Modulation of microbial resistance of *A. baumannii* isolates by Epi-Quercican™

susceptibility testing show that for the *S. aureus* clinical strains the sensitivity to E, K and DA significantly increased after Epi-Quercican™ treatment. We found that Epi-Quercican™ was effective in reducing *E. faecalis* resistance to GM, TE and S. Exposure to Epi-Quercican™ decreased *E. coli* resistance to most tested antibiotics as: TIC, TE, CIP, NET, CXM, GM, TZP and ATM. The *E. cloacae* strains susceptibility was lower for several antibiotics (20 to 30% range), in particular to CAZ (50% decrease in resistance). The most resistant pathogens- tested *A. baumannii* clinical strains were the least sensitive to Epi-Quercican™ modulating activity, but the important fact was the significant reduction of resistance to carbapenems, mainly to ATM. Our study demonstrates that Epi-Quercican™ can be used to modulate pathogens resistance to various antibiotics. We observed that the change of susceptibility to antibiotics depends on the level of resistance and the genus of bacteria. Earlier study by Harakeh et al. (2013) on modulation of the antimicrobial resistance by Epi-Quercican™ in bacteria isolated from dairy products exposed to select antibiotics showed the highest percentage increase in antimicrobial susceptibility of *E. coli*, *Salmonella* sp. and *Yersinia* sp. for gentamicin followed by cefotaxime (Harakeh et al., 2013). The percentage increase in susceptibility to clindamycin in the

presence of Epi-Quercican™ was not significant in *S. aureus*, but it was significant in *L. monocytogenes*. The increase of *L. monocytogenes* susceptibility to gentamicin was not significant.

In our study, we observed that Epi-Quercican™ exposure resulted in the highest increase in susceptibility to gentamicin for *E. coli* isolates, but not for *E. cloacae*.

An interesting study on the use of dietary supplements, such as ascorbic acid with antibiotics in treating infectious diseases was conducted by Abbas (2012). He investigated the synergy between antibiotics and each of N-acetylcysteine, ambroxol and ascorbic acid against *P. aeruginosa* clinical strains. His study showed a synergistic effect of N-acetylcysteine with β-lactam antibiotics, tetracycline in 100% of isolates, with chloramphenicol in 80% and with gentamicin in 60% of isolates. The antagonism was observed with gentamicin in 20% of isolates. Combinations of N-acetylcysteine and ambroxol showed the highest synergy with each of cefepime, ceftazidime, cefoperazone and meropenem and those of tetracycline. The synergy for scorbic acid was found with chloramphenicol in 60% of isolates and with meropenem, cefepime and cefoperazone in 20% of *P. aeruginosa* isolates. The combination of ascorbic acid with ceftazidime, levofloxacin, gentamicin and tetracycline, showed the synergistic effect in all bacterial isolates (100%), with cefoperazone, cefepime and meropenem in 80% and with chloramphenicol in 40% of isolates. One of the major constituents of Epi-Quercican™- is epigallocatechingallate which according to Kurinčič at al. has a good modulatory activity over the extrusion across the outer membrane of the macrolides such as erythromycin, azithromycin, clarithromycin, dirithromycin and tylosin, both in sensitive and resistant *Campylobacter* isolates (Kurinčič et al., 2012). According to these authors, EGCG modifies *Campylobacter* multidrug efflux systems and thus could have an impact on restoring macrolide efficacy in resistant strains. Stapleton at al. (2004) proved that aqueous extracts of Japanese green tea (*Camellia sinensis*) are able to reverse beta-lactam resistance in methicillin-resistant MRSA. Minimum inhibitory concentration (MIC) values for oxacillin were reduced from 256 and 512 to 1-4 mg/l, respectively, in the presence of these polyphenols. The modulation of beta-lactam resistance by ECG significantly enhanced the activities of flucloxacillin and the carbapenem anti-biotics imipenem and meropenem against MRSA isolates. According to Silva and Fernandes Júnior (2010) review article, the mechanisms of antimicrobial activity of natural compounds can be diverse, such as involving disintegration of the cytoplasmic membrane, destabilization of the proton motive force (PMF), electron flow, active transport and coagulation of the cell content. In the case of epicatechins, the mechanism of action is strictly connected with the disruption of membrane function (Silva and Fernandes Júnior, 2010). The impact of active plant metabolites on the bacterial cells is mainly dependent on the differences in the structure of Gram-positive and negative bacteria cell walls. It is known, that in the case of Gram-negative bacteria, the outer membrane disintegration can release the lipopolysaccharide (LPS) increasing permeability of the cytoplasmic membrane. These factors can also affect the degree and characteristics of modulation of bacterial resistance to antibiotics by natural plant components.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Standardization of inoculation technique of sugarcane smut (*Ustilago scitaminea*) for evaluation of resistance

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Different inoculation techniques *viz.* inoculation of sets by dipping in spore suspension, bud inoculation with hypodermic syringe, bud wrapping by cotton swab dipped in smut suspension and inoculation of underground bud at the time of tillering (end May) were tested to screen against sugarcane smut in the field as well as in the laboratory conditions. In the field, out of the tested inoculation techniques, the maximum disease incidence (60.63%) was observed when buds were inoculated with hypodermic syringe and minimum (8.55%) in inoculation of underground buds at the time of tillering stage. The result showed that inoculation through mechanical injury significantly increase disease incidence but at the same time also affect the bud germination (15.56%). Significant difference in smut incidence was also observed when the inoculation was carried by teliospores and sporidia separately.

Key words: *Ustilago scitaminea*, inoculation technique, screening, resistance, sugarcane.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is attacked by more than two hundred and forty diseases caused by fungi, bacteria, viruses, phytoplasmas and nematodes in India (Rott et al., 2000). Of these, fungal disease like red rot, smut and wilt are of major importance and are mainly responsible for reducing the yield and deterioration of the juice quality (Chona, 1956; Waraitch and Kumar, 1984). Sugarcane smut caused by *Ustilago scitaminea* Sydow is considered as an important disease next to red rot. The basidiomycetous fungus belongs to the order Ustilaginales and family Ustilaginaceae first described in 1870 as *Ustilago sacchari* by Mundkur (1939). Sydow (1924) opined that the smut fungus of sugarcane is distinct from *U. sacchari* and called it *U. scitaminea*. As the pathogen attacks only the meristematic tissues, it is generally referred to as a primitive parasite and it is main problem of tropical India, but now it is also becoming a problem on some varieties in North India. The disease is prevalent in all the countries that lie between 20° N and 20° S (Martin et al., 1961). The pathogen produces abundant tiny brownish black, echinulate, spherical spores ranging from 5.2 to 8.5 µm in diameter. Teliospores of *U. scitaminea* are shed from the whip and disseminated through the wind. The maximum dispersal of spores occurred at 24-27°C and 50-60 percent relative humidity (RH). The characteristic symptoms of the smut are the dark brown, whip-like fungal sorus that develops from the apex of infected stem (Butler, 1918). The wind borne spores are

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spread in the standing cane fields and can infect newly planted setts in the soil. The infection take place through the buds that may soon develop into whips; but the mycelia may remain dormant, and the use of such infected stalks as seed cane spread the disease. Whip development is determined by the season as well as the age and physiological condition of the crop. In India, many superior varieties such as CoS 510, Co 419, Co 453, Co 740, Co 975, Co 1158, Co 62175 and Bo11 have gone out of cultivation due to attack of this disease. The first epidemic of smut occurred during 1942-43 in Bihar and affected 66% of cane area (Chona, 1956; Alexander, 1986). In Karnataka during 1947-48, smut severity was so high, particularly on Co 419 that the disease had to be contained by banning ratoons crop (Subramanian and Rao, 1951). Currently, the disease has established in all the sugarcane growing states of the country especially Maharastra, Andhra Pradesh, Tamil Nadu, Uttar Pradesh, Bihar and Orrisa. Due to free movement of sugarcane from the neighboring states to Punjab, the disease incidence up to 0.8% was noticed in year 2006 (Anonymous, 2007). However, the risk of smut incursion in the state is high as un-recommended varieties grown in the state are susceptible to the disease.

Among the management strategies the best option for long term control of smut is the use of resistant cultivars. Traditional method of screening sugarcane cultivars for resistance to smut is time consuming, require large area and result are available after a long period (6-18 months). Thus, evaluation for smut resistance is commonly delayed and at the end of breeding cycle which is usually of 6-8 years, only few selections are left. A simple and rapid screening method, therefore, is necessary for evaluation of large number of progenies during preliminary selection cycles of a breeding program to expedite the development of smut resistant genotypes.

MATERIALS AND METHODS

Testing of different inoculation techniques of sugarcane smut

Field evaluation

Field experiment on testing of four inoculation techniques with two types of spores (teliospores and sporidia) of *U. scitaminea* was conducted at University Seed Farm, Ladhowal, Punjab Agricultural University, Ludhiana, India.

One hundred and thirty five single budded sets of mid-season sugarcane smut susceptible variety CoJ 88 were used for each method of inoculation. Planting of sets was carried in three rows of 4.0 m length at 0.75 cm depth in each plot. Three replications were maintained for each treatment.

Two types of smut spores (teliospores and sporidia, 1×10^6/mL^{-1}) were used for inoculation. A suspension of freshly collected teliospores was made in sterile water. Viability of the smut spores was tested and a collection showing a viability of more than 70% was used for the inoculation. For culturing of smut sporidia, teliospores collected from infected canes were dusted on Potato Dextrose Agar medium (Peeled Potato- 250 g, Dextrose- 20 g, Agar-agar powder- 20 g and 1000 mL distilled water) in test tubes and incubated for 10 days at 22 ± 2°C. After 10 days of incubation, sporidia were collected and desired concentration of sporidia (1×10^6 spores/mL^{-1}) was prepared by using sterile distilled water and used for inoculation studies as follows:

1. Inoculation of setts by dipping in spore suspension: Single budded sets of sugarcane variety CoJ 88 were inoculated by dipping in teliospores/sporidia suspension for 30 min and incubated for 24 h before sowing.
2. Bud inoculation with hypodermic syringe: Cane buds were inoculated with teliospores/sporidia with the help of hypodermic syringe. Each bud was injected with 0.5 mL spore suspension (1×10^6 spores/mL^{-1}).
3. Bud wrapping by cotton swab dipped in smut suspension: A thick spore suspension (5×10^6 spore/mL^{-1}) was prepared and applied with cotton swab on the buds.
4. Inoculation of underground bud at the time of tillering (ended May): Tillering was started in the last week of May and soil around the germinated clumps was carefully dug out. Four to five basal leaves of mother shoot were removed carefully to expose the young buds unhurt and 2-3 buds were inoculated by painting the inoculum on them with the help of hairbrush. Inoculations were carried in the evening to avoid drying. Immediately after inoculation, soil around the clumps was filled and pressed slightly to avoid any uprooting of young cane plant. After the inoculation, the field was irrigated.

Germination of setts was recorded after 30 and 45 days of planting. Observations on smut incidence were recorded fortnightly starting from 1st week of June till harvesting of the crop. Rogueing of the disease clumps was done at each observation. Cumulative smut infection for the whole season was calculated as per the following formula:

$$\text{Disease incidence (\%)} = \frac{\text{Number of infected clumps in a treatment}}{\text{Total number of germinated clump in a treatment}} \times 100$$

Laboratory evaluation

In vitro tissue culture raised young plants of a variety CoJ 88 were procured from Biotechnology Section of the Department of Plant Breeding, Genetics and Biotechnology. Plantlets were scrapped by scalper and then the plantlets were inoculated with teliospores and sporidia (1×10^6 spore/mL^{-1}) separately with the help of hairbrush. Inoculated plantlets were transformed in a long jar containing MS medium (Stock no. I- 50 ml; Stock no. II- 20 ml; Stock no. III- 20 ml; Stock no. IV-10 ml; Stock no. V - 10 ml; Sucrose- 30 g; Anasitol - 100 mg; Sterilized water 1000 mL). Plantlets were inoculated with 0.5 µL sterile distilled water served as control. All plantlets were maintained in growth room at 25±1°C and observed regularly up to three months for smut sori (sorus like spore mass of the fungus) development.

RESULTS AND DISCUSSION

Testing of different inoculation techniques for sugarcane smut

Field evaluation

Out of four inoculation techniques, inoculation of sets by dipping in spores suspension gave maximum sett germination of 77.03 and 76.66% after 30 and 45 days of sowing respectively (Table 1). The minimum set germination of 62.46 and 64 .81% was observed after 30

Table 1. Effect of different smut inoculation techniques on sett germination

Inoculation technique	Per cent sett germination					
	After 30 days			*After 45 days*		
	By Teliospores	By Sporidia	Mean	By Teliospores	By Sporidia	*Mean*
Inoculation of setts by dipping in spore suspension	76.29* (60.87)	77.77 (61.86)	77.03 (61.37)	77.03 (61.34)	76.29 (60.84)	76.66 (61.09)
Buds inoculation with hypodermic syringe	62.22 (52.07)	62.70 (52.93)	62.46 (52.50)	63.70 (53.02)	65.92 (54.27)	64.81 (53.64)
Buds wrapping by cotton swab dipped in smut suspension	71.84 (57.97)	71.84 (57.95)	71.84 (57.96)	72.58 (58.43)	71.84 (58.00)	72.21 (58.21)
Inoculation of underground buds at time of tillering stage	71.10 (57.47)	74.81 (59.88)	72.97 (58.67)	73.33 (58.89)	73.32 (58.93)	73.33 (58.91)
Mean	70.36 (57.09)	71.78 (58.16)		71.66 (57.92)	71.84 (58.01)	
CD (p=0.05) level for						
Inoculation technique		2.08			3.57	
Types of spores (Teliospores and sporidia)		NS			NS	
Inoculation techniques × types of spores		NS			NS	

Figure within parentheses represent arc sine transformed values and CD is applicable to these only. *Average sett germination of three replications.

Table 2. Effect of different smut inoculations techniques on smut incidence.

Inoculation technique	Percent smut incidence		
	Teliospores	Sporidia	*Mean*
Inoculation of setts by dipping in spore suspension	54.84* (47.76)	33.07 (35.02)	43.95 (41.39)
Buds inoculation with hypodermic syringe	69.78 (56.64)	51.48 (45.84)	60.63 (51.24)
Buds wrapping by cotton swab dipped in smut suspension	55.02 (47.88)	34.15 (35.73)	44.58 (41.80)
Inoculation of underground buds at time of tillering stage	12.12 (20.36)	4.99 (12.79)	8.55 (16.58)
Mean	47.94 (43.16)	31.17 (32.34)	

CD (p=0.05) level for: Inoculation technique = 3.20; Types of spores (teliospores and sporidia) = 2.26; Inoculation techniques × types of spores = NS; *Average disease incidence from June to 15th February 2007. Figure within parentheses represents arc sine transformed values and CD is applicable to these only.

and 45 days of sowing respectively, in bud inoculated with hypodermic syringe. No significant difference was observed when the sets were dipped in teliospores or sporidia. Similarly no significant correlation was observed between inoculation techniques and types of spores.

The maximum disease incidence (60.63%) was observed when the buds were inoculated with hypodermic syringe and minimum incidence (8.55%) was recorded in inoculation of underground bud at the time of tillering stage (Table 2). The disease incidence of 44.58% was recorded when the buds were wrapped by cotton swab dipped in smut spore suspension and 43.95% in inoculation of sets by dipping in spore suspension.

Significant difference was observed in smut incidence (%) when the buds was inoculated by hypodermic syringe and inoculation of underground bud at time of tillering. Significant difference in smut incidence was also observed when the inoculation was carried by teliospores and sporidia separately. The mean disease incidence was 47.94% when sets were inoculated

with teliospores and it was only 31.17% by sporidial inoculation (Table 2). Inoculation is through mechanical injury, that is, hypodermic syringe, increase smut disease incidence and decrease in the germination as compared to inoculation without injury. Waller (1970) made a pioneering work in comparing different methods of smut inoculation and found that injection inoculation may induce greater smut infection than dip inoculation which is confirmation of our study. Olweny et al. (2008) critically evaluated the smut inoculation techniques in sugarcane seedlings and explored the possibility of screening for smut resistance at the seedling stage. Dalvi et al. (2011) also used artificial inoculation of smut sori to sugarcane sets by dipping into smut spore suspension for 30 min and planted in field for screening of somaclones and achieved significant results. The findings of other workers (Luthra et al., 1938; Waraitch and Kumar, 1987; Duttamajumder, 2000) are also in accordance with our study.

Duttamajumder (2000) reported that inoculation of underground buds at the time of tillering gave 83% disease incidence as compared to 36% in dipping of setts in spore suspension but the present study negates their observations.

Laboratory evaluation

In tissue culture raised plantlets, no smut sori were developed. However, mycelium of the fungus was present in the leaves, which is not a true indication of disease development in this experiment Therefore, this method is not to be considered as a substitute for field screening which is more effective. Singh et al. (2005) tried smut screening on tissue cultured sugarcane plantlets and reported that the method is not a substitute for field screening. The present study also did not give any positive direction towards the quick screening technique.

Conflict of Interests

The authors have not declared any conflict of interests.

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Epidemiology of bovine tuberculosis in Butajira, Southern Ethiopia: A cross-sectional abattoir-based study

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A cross-sectional study was conducted at Butajira Municipality abattoir from December, 2009 to April 2010 to investigate the epidemiology of bovine tuberculosis (bTB) in Butajira, Southern Ethiopia. Postmortem examination, mycobacterial culturing and multiplex genus typing techniques were used. An overall prevalence of 9% (40/446) of the animals examined harbor gross tuberculous lesions up on detailed post-mortem examination. Statistically significant difference was observed in the prevalence of bTB between different age groups (χ^2 = 11.441, p= 0.003) and body condition scoring (χ^2 = 10.384, p = 0.006). Higher prevalence of bTB was observed in older animals and animals with poor body condition. Bacteriological culture of the 40 samples gave growth on 13 with 9 of them acid fast Bacilli (AFB) positive. Genus typing of the AFB positive isolates by multiplex polymerase chain reaction (m-PCR) revealed seven non-tuberculous mycobacterium (NTM) and 1 *Mycobacterium tuberculosis* complex (MTBC) isolates. Further characterization of the isolates at specific species and investigation of the disease is recommended for controlling it in livestock and safeguard public health.

Key words: Abattoir, bovine tuberculosis, Butajira, multiplex genus typing, epidemiology, Ethiopia, postmortem examination, prevalence.

INTRODUCTION

Bovine tuberculosis (bTB) is a chronic infectious disease of animals characterized by the formation of granulomas in tissues and organs. It is caused by slowly growing non-photochromogenic bacilli members of the *Mycobacterium tuberculosis* complex (MTBC): *M. tuberculosis, Mycobacterium africanum Mycobacterium microti, Mycobacterium bovis, Mycobacterium canetti* and *Mycobacterium caprae* species (Radostits et al., 2000; Thoen et al., 2006).

bTB has been significantly widely distributed

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Abbreviations: AFB, Acid fast Bacilli; **bTB,** bovine tuberculosis; **m-PCR**, multiplex polymerase chain reaction; **MTBC,** *Mycobacterium tuberculosis* complex; **NTM,** non-tuberculous mycobacterium.

throughout the world and has been a cause for great economic loss in animal production and the most frequent cause of zoonotic TB in man (Tenguria et al., 2011). Moreover; in developing countries, still constitutes a major threat to public health where surveillance and control activities are often inadequate or unavailable (Ayele et al., 2004). bTB in humans is becoming increasingly important in developing countries, as humans and animals are sharing the same micro-environment and dwelling premises, especially in rural areas. Moreover, due to the association of mycobacteria with the high prevalence of HIV/AIDS in the developing world and susceptibility of HIV/AIDS patients to tuberculosis in general, the situation change is most likely (Amanfu, 2006).

bTB is one of the endemic infectious diseases that have long been recorded in Ethiopia (FAO, 1967; Hailemariam, 1975). Reports of abattoir based surveys showed varied prevalence that range from 1.48 to 15.4% in different municipal and export abattoirs of the country (Asseged et al., 2004; Teklu et al., 2004; Shitaye et al., 2006). However, still, there is lack of knowledge on the actual prevalence and distribution of the disease at a national level. Accurate and sound scientific baseline prevalence data of bTB across a range of eco-epidemiological settings is needed to ensure public health policies and disease control strategies. Thus, the study was carried out in Butajira Manucipital abattoir, Meskan district of the Southern Ethiopia to determine the epidemiology of bTB in slaughtered cattle.

MATERIALS AND METHODS

Study area and animals

The study was conducted from December 2009 to April 2010 in Butajira municipality abattoir, Meskan Woreda, Southern Nations Nationalities and Peoples Regional State (SNNPR) in Ethiopia. The area is located 130 km south of Addis Ababa, with Butajira being the main town. It has varying climates zones from arid dry lowland areas around 1500 m.a.s.l altitude to cool mountainous areas above 2000 m.a.s.l. The area is rich in livestock population; 123,495 bovine, 2,532 ovine, 19,231 caprine and 10,475 equine (CSA, 2004). According to the available logistics and time, a total of 446 apparently normal animals slaughtered in the abattoir from December 2009 to April 2010 were included in the present study. The major sources of cattle to this abattoir were Sulte, Enceno, Makecho, Threeamba and Draama.

Study design and sampling techniques

A cross-sectional study was designed to determine the prevalence and assess the potential risk factors of bTB. Tissue lesion samples suspected to be positive for bTB were sampled aseptically from suspicious organs.

Individual animal identification number, place of origin, breed, sex, ante-mortem examination findings were recorded at the animal quarantine stations before submission to slaughter houses. Age categorization was made using dental eruption and wear as described by Amstutz (1998). Physical examination of animals including body condition scores, history of animals, age groups were carried out before they were slaughtered. Body condition scoring was done using the method developed for zebu cattle (Nicholson and Butter worth, 1986). Parameters like sex, age, origin and body condition score were assessed for the presence of possible association with the presence of TB lesion.

Post-mortem examination

Post mortem examination was done as described by Corner (1994). Briefly, detailed inspection of lymph nodes and organs that are reported to be frequently affected were done under bright light source. Lymph nodes were incised into slices of 2 cm using separate surgical blades in order to facilitate detection of tubercular lesions. The slices were then examined for the presence of tubercular pathological lesions (Gracey and Collins, 1992). Tissues with suspected lesions were collected separately in sterile universal bottles with phosphate buffer saline solution and kept at +4°C at the Butajira District Veterinary Clinic before being transported once per week to the TB laboratory of AHRI (Neill et al., 1992). Type and stage of tuberculosis lesion, frequency of infection of anatomical sites were also recorded for individual tuberculous suspected cattle.

Isolation and identification of mycobacteria

Specimens collected from tuberculosis suspected slaughtered cattle were processed according to OIE established standard protocols (OIE, 2004). The specimens were sectioned into pieces using sterile blades, and homogenized by pestle and mortar for 10 min. The homogenate was decontaminated by adding an equal volume of 4% NaOH for 15 min followed by centrifugation at 3000 rpm for another 15 min. The supernatant was discarded while the sediment was neutralized by 1% (0.1N) HCl using phenol red as an indicator. Neutralization was achieved when the color of the solution changed from purple to yellow (WHO, 1998). Thereafter, 0.1ml of suspension from each sample was spread onto a slope of Lowenstein-Jensen (LJ) medium. Duplicates of LJ were used; two enriched with sodium pyruvate while the other two was enriched with glycerol. Cultures were incubated aerobically at 37°C for about 5-8 weeks with weekly observation for growth of colonies (Vestal, 1998). Identification of the mycobacterial species was performed based on the rate of growth and colony morphology and growth on pyruvate or glycerol supplemented LJ media. Growth was considered when mycobacterial colony was observed and examined using the Ziehl-Neelsen technique for confirmation of AFB (de Kantor et al., 1998).

Molecular typing of isolates

AFB positive isolates were heat-killed by mixing approximately 2 loopful of colonies in 200 µl distilled H_2O followed by incubation at 80°C for 45 min. Following the standard procedure by Wilton and Cousins (1992), multiplex polymerase chain reaction (m-PCR) was used to confirm the presence of genus Mycobacterium in the isolate and to differentiate MTBC from *Mycobacterium avium* complex, and other mycobacterial species.

Data collection, management and statistical analysis

Data related to age, breed, body condition and origin of each animal were recorded on a data sheet during the ante mortem examination. Presence or absence of TB-like lesions and affected tissue(s) were recorded on postmortem examination. The recorded data were entered into Microsoft Excel data sheets and analyzed

Table 1. Association of animal risk factors with tuberculous lesions.

Risk factor	Number of examined	Number positive (%)	95% CI	χ^2	P Value
Age (years)				11.44	
<5	70	8(11.4%)	5.07-21.28		0.003
5-8	325	25(7.7%)	4.54-10.43		
>8	51	7(13.7%)	5.70-26.26		
Sex				0.32	
Male	329	29 (8.8%)	5.48-11.76		0.57
Female	117	11 (9.4%)	4.79-16.19		
Body condition				10.38	
Poor	62	9(14.5%)	6.86-25.78		0.006
Medium	284	24(8.5%)	5.49-12.31		
Good	100	7(7%)	2.86-13.89		
Origin of animals				2.26	0.68
Sulte	88	9 (10.2%)	4.78-18.53		
Inceno	119	8(6.7%)	2.95-12.82		
Draama	101	11(10.9%)	5.56-18.65		
Makecho	79	5(6.3%)	2.08-14.16		
Threeamba	59	7(11.9%)	4.91-22.93		

Table 2. Distribution of lesions in lymph nodes with their respective frequency of occurrence.

Anatomical site	Organ affected	Frequency (%)
Head	Sub-mandibular lymph nodes	3 (7.5)
	Retropharyngeal lymph nodes	1 (2.5)
Thoracic	Tracheo-bronchial Lymph nodes	14 (35)
	Mediastinal Lymph nodes	11 (27.5)
Abdomen	Mesenteric Lymph nodes	11 (27.5)
	Total	40 (100)

using SPSS 17.0 statistical software. Descriptive statistics was used to determine the proportion of cattle carcass harboring tuberculous lesions. The difference between the effects of different risk factors on prevalence was analyzed. A statistically significant association between variables was said to exist if the calculated $P<0.05$. The range and frequency of anatomical sites with tuberculous lesions were recorded for each carcass examined. For all the analysis performed, $P \leq 0.05$ was taken as statistically significant.

RESULTS

Prevalence and analysis of associated risk factors

The prevalence of animals with suspicious tuberculous lesions was 9% (95% CI: 6.48-12.01). The association of different risk factors responsible for the occurrence of the disease is depicted in Table 1. Accordingly, statistical significant differences were observed between tuberculosis lesion prevalence and age and body.

Distribution and location of pathological lesions

The frequency and distribution of lesions according to organ level and anatomical sites is indicated in Table 2. 62.5% (25/40) of the total gross lesions observed was from the lymph nodes of thoracic region followed by 27.5% (11/40) in the mesenteric lymph nodes and 10% (4/40) of gross lesions were detected in the lymph nodes of the head.

Mycobacteriology and microscopy

Outof thetotal40tuberculous lesions mycobacteriologically processed and cultured, growth was observed in 13 with nine of them acid fast bacilli (AFB) positive.

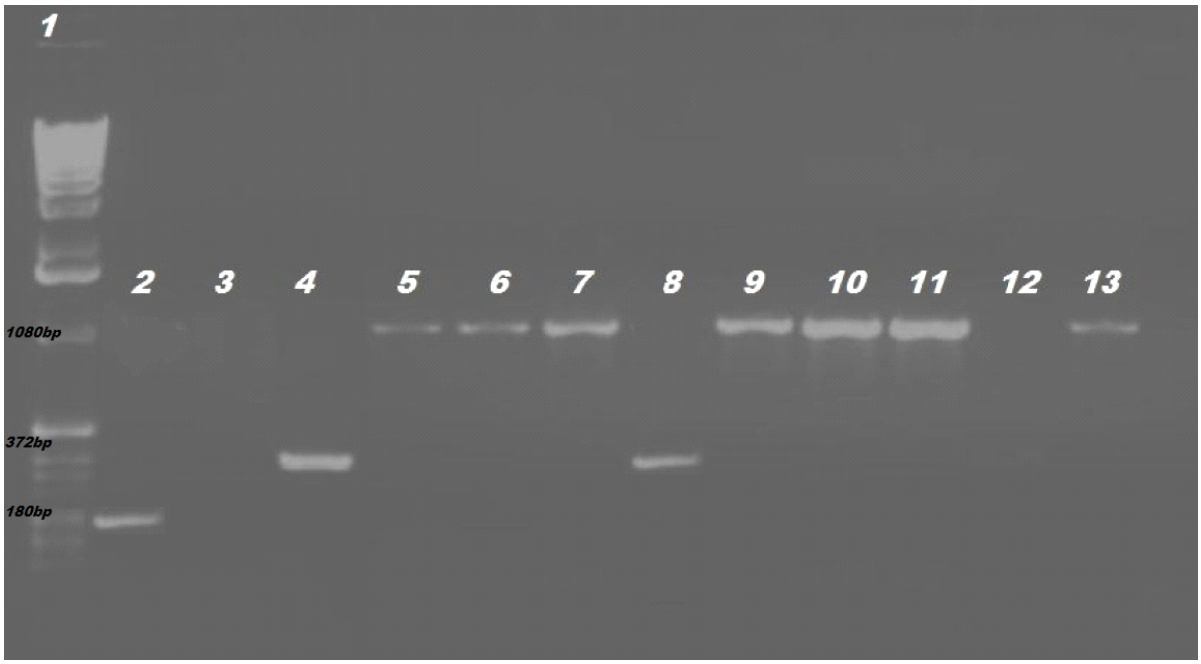

Figure 1. Gel electrophoresis separation of polymerase chain reaction products of multiplex genus typing of the genomic DNA of mycobacteria isolated from cattle with grossly suspicious TB lesions. Lane: 1 = a ladder of band at an interval of 100 bp DNA; 2 = *Mycobacterium avium* (positive control); 3 = Qiagene-water (negative control); 4 = *Mycobacterium tuberculosis* complex (positive control); Lanes 5-13 are isolates from individual cattle with tuberculous lesions; Lanes 5-7, 9-11 and 13 are positive samples for genus Mycobacterium (1080 bp); Lane 8 is positive for *Mycobacterium tuberculosis* complex (372 bp); Lane 12 is negative for the genus mycobacterium.

Genus typing of AFB isolates

Genus specific m-PCR typing of 9 AFB positive isolates showed a PCR product size of 1030 bp for 7 isolates and 372 bp for 1 isolate which is specific for NTM and MTBC, respectively. While 1 isolate did not show a signal at all (Figure 1).

DISCUSSION

The proportion of slaughtered cattle that harbor tubeculous lesions up on detailed abattoir inspection were 9%. Comparable findings were recorded by Biffa et al. (2009) and Ameni et al. (2001) that reported a prevalence of 10.1 and 8.8%, respectively. The study result was higher than the previous prevalence reports by various authors: 4.5% in Hosanna (Teklu, 2003), 5.2% in Nazreth (Ameni and Wudie, 2003), 1.48% in Addis Ababa (Asseged et al., 2004), 2.4% in Jimma (Jemale, 2005), 3.46% in Addis Ababa (Shitaye et al., 2006) and 5.8 in Setit-Humera (Romha et al., 2013). In contrarst, it was lower the finding by Mamo (2007) who reported a prevalence of 24.7%. These variations could be due to differences origin, type of production system and breed of animals slaughtered in the abattoirs (Romha et al., 2013).

In parallel to previous reports (Corner, 1994; Neill et al., 1994; Collins, 1996, Whipple et al., 1996), large proportions of tuberculous lesions (62.5%) were detected in the lymph nodes of the thoracic region. This suggests that respiratory route is the primary route of transmission and infection (O'Reilly and Daborn, 1995; Ameni and Wudie, 2003; Teklu et al., 2004).

Statistically significant difference was observed in the prevalence of bTB between different age groups (χ^2 = 11.441, p= 0.003) and body condition scoring (χ^2 = 10.384, p = 0.006) up on analysis of different risk factors. Higher prevalence of bTB had been recorded in old aged and poor body conditioned animals. As the age of the cattle increase owning to increased chances of exposure and infection with bTB, Humblet et al. (2009) explicated that those stressors, malnutrition and immuno-suppressants increases with age; thus, older animals are more likely to have been exposed than younger ones. It has been suggested that increased incidence of bTB in older animals can be explained by a declining of protective capability in aging animals (O'Reilly and Daborn, 1995). Similarly, the high prevalence of bTB in poor conditioned cattle could be due to the fact that animals under good body condition are with good immune status that can respond to any foreign protein better than those with poor body condition (Collins and Grange, 1994). Moreover, previous studies confirmed that animals with poor body conditions and in nutritional deficiency have reduced immune resistance to bTB

(Doherty et al., 1995).

The culture result of bTB suggestive pathologic lesions was low as compared to other study reports (Ameni et al., 2007, 2010). Failure to grow the major portions of the specimens could have been due to misclassification of non tuberculous lesions (Teklu et al., 2004) caused by other granuloma-causing organisms (Radostits et al., 2000). Fully calcified lesions without viable tubercle bacilli could also give the low recovery of mycobacteria (Pritchard, 1988). Multiplex genus typing of the isolates revealed that out of 9 AFB positive isolates 7 isolates showed signals for the genus mycobacterium (NTM) and 1 showed signal for mycobacterium tuberculosis complex respectively. The isolation of large number of NTM showed the importance of NTM in the epidemiology of bTB to cause tuberculous like lesions. Similar study results from different part of Ethiopia (Shimelis, 2008; Berg et al., 2009; Romha et al., 2013) and other African countries also shows the isolation of several NTM strains from animals with tuberculous lesions (Diguimbaye-Djaïbe et al., 2006; Oloya et al., 2006). Moreover, NTM had been isolated from milk and nasal swab of tuberculin reactor animals in Chifra pastoral district of Afar region, Norht eastern Ethiopia (Ashenafi et al., 2013).

The study supports the endemic nature of bTB in cattle and the potential zoonotic risk of bTB to humans in the study area. Further investigation to reveal the epidemiological significance for public health in the region and to identify the potential risk factors for infection and transmission of bTB among the livestock and at the interface of animals and humans is suggested. Education and awareness creation among community about the economic and public health significance of bTB is also important to design a feasible community-based control program.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Demographic and microbiological profile of cystic fibrosis in Durban, South Africa

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Cystic fibrosis (CF) necessitates long-term treatment with multiple antibiotics creating selection pressure for the development of antibiotic resistance in infecting and/or colonizing organisms, impacting on disease management, morbidity and mortality. Sputum samples were obtained from patients attending the only two CF clinics in Durban over a year. The patient demographics and clinical data were recorded. Bacterial isolates were subjected to identification, susceptibility testing and phenotypic screening for extended spectrum β-lactamases (ESBLs), AmpC β-lactamases and metallo-β-lactamases (MBLs). Twenty-five patients constituted the study sample. The most common genotype was F508del and the most common pathogen was *Pseudomonas aeruginosa* with susceptibility to antibiotics ranging from 14-100% with marginal differences between mucoid and non-mucoid phenotypes. All *P. aeruginosa* isolates were putative ESBL producers and 75% were putative MBL producers. The incidence, prevalence and susceptibility patterns of bacterial pathogens and colonizers isolated from cystic fibrosis patients should be closely monitored to optimize management and treatment options in a disease requiring chronic antibiotic therapy which increases the propensity for the development of antibiotic resistance.

Key words: *Pseudomonas aeruginosa*, cystic fibrosis, extended spectrum β-lactamases (ESBLs), metallo-β-lactamases (MBLs).

INTRODUCTION

Cystic fibrosis (CF) is an autosomal recessive disease caused by a mutation in the gene of the CF transmembrane regulator (CFTR) resulting in high morbidity and early mortality (Coutinho et al., 2008). In South Africa, approximately 1 in 20 individuals in the white population, 1 in 55 in the population of mixed-race and 1 in 90 black Africans carry a CFTR mutation (The South African Cystic Fibrosis Consensus Document, 2012). CFTR mutations vary considerably between populations and regions of the world with F508del constituting approximately 66% of all CF mutations globally (Saleheen and Frossard, 2008). The F508del mutation further accounts for up to 81% of all CF alleles in the South African white/caucasian population (Goldman et al., 2001), 53% in South Africans of mixed-race but is rarely detected in black African populations (Maseka et

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al., 2013).

The primary cause of long term complication and frequently death among CF patients is chronic bacterial infection of the respiratory tract with *Pseudomonas aeruginosa, Staphylococcus aureus* and *Haemophilis influenzae* as common causative bacteria (Hauser et al., 2011), although many other opportunistic bacteria have also been isolated, notably *Burkholderia cepacia* complex (BCC), *Stenotrophomonas maltophilia* and *Acinetobacter* spp., while, *Aspergillus* spp., non-tuberculosis mycobacteria and respiratory viruses have also been implicated (Saiman and Siegel, 2004). The most problematic bacterial infections are caused by *P. aeruginosa* and BCC and are characterized by significant reduction in lung function and low responsiveness to antibiotic therapy because of antibiotic resistance (Drevinek et al., 2008). Few disease states have high prevalence of antibiotic–resistant infections as does CF with 25-45% of adult CF patients estimated to be chronically infected with multi-resistant bacteria within their respiratory tracts (Cystic Fibrosis Foundation, 1994). Both bacteria also pose the risk of epidemic spread within the CF community, with the BCC being distributed among CF patients to much smaller extent (3 – 30%) as compared to *P. aeruginosa* (70 – 80%) (Drevinek et al., 2008). Neonates with CF have structurally normal lungs and no *P. aeruginosa*, but non-mucoid *P. aeruginosa* is acquired after variable time periods (LiPuma, 2010). The prevalence of *P. aeruginosa* increases with age and non mucoid *P. aeruginosa* is converted to mucoid *P. aeruginosa* which is usually associated with increasing lung deterioration with time (Govan and Deretic, 1996; Li et al., 2005).

This cross sectional observational study describes the demographic and microbiological profiles of CF in patients attending the only two dedicated CF clinics in the public and private health sectors in Durban, South Africa.

MATERIALS AND METHODS

Ethical considerations

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu Natal, South Africa (BE148/11).

Study sample

Patients attending the only two CF clinics (one public and the other private) in Durban, South Africa on scheduled clinic days over a 12 months period from June 2012-June 2013 formed the study sample and only 1 sputum sample (after expectoration) was sourced from each patient. The demographic and clinical data recorded included race, gender, age, age of first diagnosis and CFTR genotype (if known).

Microbiology

Twenty – five sputum samples were obtained (15 from the public sector and 10 from the private sector clinic). Bacterial and fungal isolates were identified and minimum inhibitory concentrations (MICs) for the bacterial isolates were determined using the VITEK MS and the VITEK 2 systems (bioMérieux, USA) with MICs analyzed according to CLSI guidelines (CLSI, 2012) for benzyl penicillin, oxacillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefuroxime, cefotaxime, cefoxitin, ceftazidime, cefepime, ertapenem, imipenem, meropenem, amikacin, gentamicin, tobramycin, ciprofloxacin, moxifloxicin, erythromycin, clindamycin, teicoplanin, vancomycin, tetracycline, tigecycline, fusidic acid, mupirocin, nitrofurantoin, colistin and trimethoprim/ sulfamethoxazole appropriately. *Streptococcus pneumoniae* ATCC 49619 and *Enterococcus faecalis* ATCC 29212 served as controls for Gram-positive identification and susceptibility respectively, *Shigella sonnei* ATCC 25931 and *Escherichia coli* ATCC 25922 served as controls for Gram-negative identification and susceptibility respectively and *Candida albicans* ATCC 14053 was the control for fungal identification.

All isolates were screened for extended-spectrum β-lactamase (ESBL) production using the double disc synergy method (Begum et al., 2013), AmpC β-lactamase production using the cefoxitin disc sensitivity test, inducible AmpC β-lactamase production using the disk antagonism test (Upadhay et al., 2010) and metallo-β-lactamase (MBL) production using the imipenem-EDTA combined disk test (Yong et al., 2002).

RESULTS

Twenty-five out of a total of 42 patients currently registered with the CF clinics constituted the study sample, representing 60% of the known patient cohort. There were 14 adults and 11 children, 14 males and 11 females, ranging in age from 2-33 years. Twenty-three (92%) of patients were white, 1 was of mixed-race and 1 was Indian. Of the 23 white patients, 18 were homo-zygous and 2 heterozygous for the F508del mutation, one showed the 3659 del C, 1 showed 1 copy each of the ∆1507 and 1 the E60X mutations and 1 was unknown. The CFTR genotypes of the other patients were unknown. Most of the patients were diagnosed in infancy or *in utero*. Only 1 patient was diagnosed in adulthood (Table 1).

Twenty-two bacterial and 6 fungal isolates were identified (Table 1). Patients harboured 3 different *Candida* spp. and 6 bacterial species other than the mucoid and non-mucoid *P. aeruginosa* in 12 different permutations. *P. aeruginosa* constituted the vast majority of bacterial isolates at 15 (68%).

The antibiotic susceptibility of *P. aeruginosa* isolates is shown in Table 2 with a marginal difference in susceptibility between the mucoid and non-mucoid phenotypes. *E. cloacae* was resistant to ampicillin, amoxicillin/clavulanate and cefoxitin; *K. pneumonia* was resistant to amoxicillin /clavulanate, cefoxitin and tobramycin; *B. cepacia* was resistant to meropenem and ciprofloxacin, *S. aureus* was resistant to benzyl penicillin and rifampicin and *Streptococcus mitis* showed intermediate resistance to erythromycin. All isolates were susceptible to colistin, used against multi-drug resistant isolates as a last resort because of its nephrotoxicity. All 20 (100%) Gram-negative isolates yielded a positive test for ESBLs and 15

Table 1. Patient demographics, age of CF diagnosis, CFTR genotype and microorganisms isolated from sputum.

Patient	Age	Gender	Race	Age of diagnosis	Genotype	Microorganisms Isolated from Sputum
1	2	M	W	Birth	Homozygous F508del	Normal respiratory tract bacterial flora
2	3	M	C	1 Year	Unknown	Normal respiratory tract bacterial flora; *Candida dubliniensis*
3	3	F	W	Birth	Homozygous F508del	Normal respiratory tract bacterial flora
4	4	F	W	1 Year	homozygous F508del	Normal respiratory tract bacterial flora; *C. dubliniensis*
5	5	F	W	Birth	homozygous F508del	Normal respiratory tract bacterial flora
6	7	M	W	3 months	homozygous F508del	*S. mitis S. maltophilia; Candida albicans*
7	8	F	W	13 Months	homozygous F508del	Normal respiratory tract bacterial flora
8	8	M	W	Birth	homozygous F508del	Normal respiratory tract bacterial flora
9	10	M	W	17 months	F508del /G551D	Mucoid *P. aeruginosa*
10	10	M	W	1 week	homozygous F508del	Non-mucoid *P. aeruginosa*
11	12	F	W	3 months	homozygous F508del	Non Mucoid *P. aeruginosa*
12	15	M	W	*In vitro*	Unknown	Mucoid and non-mucoid *P. aeruginosa; E. cloacae; C. albicans*
13	16	M	W	13 months	3659 del C	Mucoid and non-mucoid *P. aeruginosa*
14	18	F	W	22 months	homozygous F508del	*E. cloacae*
15	19	F	W	*In vitro*	homozygous F508del	Mucoid *P. aeruginosa; K. pneumonia; C. albicans*
16	20	M	W	Birth	Heterozygous F508del	Mucoid *P. aeruginosa*
17	21	F	W	9 months	homozygous F508del	Mucoid and non-mucoid *P. aeruginosa*
18	21	M	W	1 week	homozygous F508del	Normal respiratory tract bacterial flora
19	24	M	W	6 weeks	homozygous F508del	Normal respiratory tract bacterial flora; *C. albicans*
20	25	M	I	4 years	Unknown	*S. aureus*
21	25	F	W	1 month	∆1507; E60X (1 copy each)	*B. cepacia*
22	26	F	W	*In vitro*	homozygous F508del	Non-mucoid *P. aeruginosa*
23	28	M	W	17 months	homozygous F508del	Mucoid *P. aeruginosa*
24	32	F	W	20 years	homozygous F508del	Normal respiratory tract bacterial flora
25	33	M	W	1 year	homozygous F508del	Mucoid and non-mucoid *P. aeruginosa; Candida glabrata*

F- female; M- male; I- Indian; C- mixed race; W- white.

Table 2. Susceptibility of *P. aeruginosa* isolates to selected antibiotics.

Antibacterial agent	Susceptibility (%)	
	Mucoid *P. aeruginosa* (n= 8)	Non-mucoid *P. aeruginosa* (n= 7)
Piperacillin/-tazobactam	6 (75)	7 (100)
Ceftazidime	7 (88)	7 (100)
Cefepime	6 (75)	6 (86)
Imipenem	8 (100)	6 (86)
Meropenem	7 (88)	7 (100)
Amikacin	4 (50)	3 (43)
Gentamicin	2 (25)	1 (14)
Tobramycin	7 (88)	5 (71)
Ciprofloxacin	6 (75)	4 (57)
Colistin	8 (100)	7 (100)

(75%) were positive for MBLs. Although 18 (90%) of isolates screened were positive for AmpC β-lactamase production on the basis of resistance to cefoxitin on the disc sensitivity test, none were inducible according to the

Table 3. Results of phenotypic screening for β-lactamases.

β-lactamase	*P. aeruginosa* (n=15)	*E. cloacae* (n=2)	*K. pneumonia* (n=1)	*B. cepacia* (n=1)	*S. maltophilia* (n=1)
ESBL	15	2	1	1	1
AmpC	13	2	1	1	1
Inducible AmpC	0	0	0	0	0
MBL	12	1	0	1	1

disk antagonism test (Table 3).

DISCUSSION

Although CF occurs in all South African population groups, it is better described in the white and mixed race populations while its prevalence in the black population is less well known (Westwood et al., 2006) indicating potential under-diagnosis as the black population group comprises greater than 80% of the total KwaZulu Natal population. Notwithstanding the fact that CF patients may be managed outside of the two CF clinics, possible under-diagnosis may be attributed to CF being omitted from differential diagnoses in this population group, poor access to medical care and misdiagnosed as malnutrition, indicative of CF, is very common in the black population for poverty-related reasons (Maseka et al., 2013). Further, just two of the 13 CF clinics in South Africa are located on the coast in Durban and may not be easily accessible to people from the inner and rural areas. The predominant CFTR genotype was F508del as recorded previously for the South African population (Maseka et al., 2013).

Many organisms that are isolated from sputa of CF patients are pathogens (e.g. *S .aureus*) that often progress to colonize the upper respiratory tract or are common environmental organisms that behave as opportunistic pathogens (e.g. *P. aeruginosa*) (Valenza et al., 2008; Cardoso et al., 2008). *S. aureus* is usually the first pathogen to infect and colonize the airways of CF patients (Hauser et al., 2011), while *P. aeruginosa* occurs in early childhood with prevalence increasing with age such that as many as 80% of patients with CF are infected with *P. aeruginosa* by the time they reach the age of 20 (Li et al., 2005). The median age recorded in this study was 16 explaining the predominant isolation of *P. aeruginosa*.

Patients with CF are at risk of multi-resistant infections as a result of endo-bronchial bacterial infections that in most cases cannot be eradicated (Aaron, 2007) and frequent high dose antibiotic therapy is an essential part of CF management. Patients are exposed to multiple courses of antibiotics both chronically and intermittently, and this introduces selective pressure for the development of antibiotic resistance in infecting and/or colonizing organisms (The South African Cystic Fibrosis Consensus Document, 2007).

In comparison, non-mucoid *P. aeruginosa* showed lesser susceptibility to imipenem, ciprofloxacin and the aminoglycosides while mucoid *P. aeruginosa* were less susceptible to meropenem, the cephalosporins and the piperacillin-tazobactam inhibitor combination. Although differences in antimicrobial susceptibility between mucoid and non-mucoid *P. aeruginosa* have been documented in many studies, the significance is yet to be ascertained. Notwithstanding the marginal differences in susceptibility observed in this study, it is postulated that the exo-polysaccharide/alginate compromises access to antibiotics such that the mucoid isolates are exposed to sub-inhibitory concentrations of antibiotics facilitating the evolution of resistance (Hauser et al., 2011).

All *P. aeruginosa* isolates in this study were putative ESBL producers and were resistant to most of cephalosporin generations. Infections with ESBL-producing pathogens occur in patients who have recently received broad spectrum antibiotics, particularly third-generation cephalosporins and quinolones as is the case with chronic therapy in CF. Multi-drug resistance to the aminoglycoside, fluoroquinolone and β-lactam antibiotic classes was also evident and attributed to the co-carriage of resistance genes on the same genetic determinants of resistance, whether plasmids, transposons or integrons, severely limiting treatment options (Kanj and Kanafan, 2011).

The incidence, prevalence and susceptibility patterns of different microorganisms in the sputa of CF patients should be closely monitored to optimize management and treatment options in a disease requiring chronic antibiotic therapy to reduce morbidity and mortality. The complexity and diversity of β-lactamase expression in *P. aeruginosa* from CF patients, necessitates early detection to inform efficacious antibiotic therapy as antibiotic options are limited not only in the treatment of CF but in the treatment of all infections globally.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Isolation and characterization of bacterial symbiont *Photorhabdus luminescens* SL0708 (Enterobacteriales: Enterobacteriaceae)

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***Photorhabdus* spp. bacteria are associated with infective juveniles of entomopathogenic nematodes of the *Heterorhabditidae* family. After entering the host, these bacteria cause septicemia and kill the insect within 48-72 h. The objectives of this study were to isolate and identify the symbiont bacteria of the *Heterorhabditis* sp. SL0708 entomopathogenic nematode, isolated in Valle del Cauca, Colombia. To this end, we performed genotypic, phenotypic and biochemical tests and assessed its pathogenicity using *Galleria mellonella* larvae. The *Photorhabdus luminescens* SL0708 bacterial strain that we isolated from nematode SL0708 had the Gram-negative bacillus morphology characteristic of *P. luminescens* species; after 72 h of incubation, the bacterial colonies were convex, slimy, shiny and small (1 mm in diameter). Biochemical assays showed that the *P. luminescens* SL0708 strain had properties similar to those described for *Photorhabdus* sp. to date. However, *P. luminescens* SL0708 differed from the other subspecies of *P. luminescens* by its ability to oxidize arabinose, the microorganism has the necessary enzymes to oxidize and transform the monosaccharide into energy, using it as a carbon source. The phylogenetic tree constructed in this study reveals that *Photorhabdus* sp. SL0708 strain belongs to the species *P. luminescens,* establishing a close group of *P. luminescens hainanesis.* Ultimately, last instar larvae of *G. mellonella* were susceptible to *P. luminescens* SL0708, with significant differences between treatments.**

Key words: Colombia, 16S rRNA, phylogeny, infective juveniles, entomopathogenic nematodes, *Heterorhabditis* sp.

INTRODUCTION

Photorhabdus sp. bacteria are characterized as Gram-negative bacilli of the enterobactereacea family that have a symbiotic relationship with nematodes of the genus *Heterorhabditis* Poinar (Rhabditida: Heterorhabditidae) and have bioluminescent properties (Boemare and Akhurst, 2006). The bacteria inhabit the intestinal lumen of the infective juveniles (IJs) that transport the bacteria from one host to another, providing them with protection

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from environmental conditions. The IJs enter their host through the mouth, anus, spiracles and cuticle using the tooth on the forepart of their bodies. Once in the hemocoel, the IJs regurgitate the symbiotic bacteria, which cause sepsis and the subsequent death of the insect within 48-72 h after entering the host. The insect tissues are then degraded and provide an ideal environment for nematode growth and reproduction. The IJs feed; develop to adult and reproduce (Adams et al., 2006; Waterfield et al., 2009). *Photorhabdus* sp. produces toxins that contribute towards insect lethality, as toxin complexes Tcs (Lang et al., 2014). Several of the toxins produced by the bacterial symbiont protect the cadaver microenvironment due to their antimicrobial, antihelmintic and insecticidal activities (Orozco et al., 2013). The IJs then retrieve the bacteria and leave the insect cadaver in search of a new host (Stock et al., 2012).

At present, three species of *Photorhabdus* have been reported: *P. luminescesns* Thomas and Poinar, *Photorhabdus temperata* Fischer-Le Saux, *Photorhabdus asymbiotica* Akhurst and seven subspecies have been specifically described for *Photorhabdus luminescens* (Singh et al., 2012). These species and subspecies' characterization and identification have been accomplished using colony phenotypic characteristics and employing biochemical and molecular technologies such as amplification of the 16S rRNA gene (Ruisheng and Grewal, 2010).

Even though there are many studies in Colombia related to heterorhabditids isolation for their use as potential biological control agents in different crops (López et al., 2007), studies on their bacterial symbionts have not been reported. Therefore, in this study, we set out to isolate, identify and evaluate the pathogenicity of the symbiotic bacteria of the *Heterorhabditis* sp.SL0708 entomopathogenic nematode, isolated from the Valle del Cauca, Colombia.

MATERIALS AND METHODS

Bacterial isolation

The bacterial strain was isolated from larvae of *Galleria mellonella* L (Lepidoptera: Pyralidae) infected 48 h earlier with *Heterorhabditis* sp. SL0708. The bodies were superficially disinfected with sodium hypochlorite 0.1% for 1 min and washed with sterile water for 3 min. A drop of hemolymph was extracted by puncturing the first larval intersegment and inoculated on NBTA (nutrient agar supplemented with 0.004% (w/v) bromothymol blue and 0.0025% (w/v) of triphenyltetrazolium chloride) and MacConkey agar for 48h at 25°C. After 48 h of incubation, we analyzed the light output of the colonies in a dark room for 15 min. Only the colonies that were luminescence were used in this study.

Phenotypic characterization

After 72 h of incubation at 25°C on nutrient agar, MaConkey, NBTA and TSA (diffusible pigments), morphological properties (shape, color and size) of the selected colonies were evaluated and compared with the following isolates: *P. luminescens* subsp. *luminescens* Poinar, *P. luminescens* subsp. *akhurstii* Akhurst and *P. temperata* subsp. *temperata* Fischer-Le Saux. To evaluate the optimum growth temperature, 40 mL of nutrient broth with the bacterial inoculum were prepared by triplicate; the liquid cultures were incubated at temperatures of 25, 30 and 35°C; we observed growth after 48 h. We then extracted an aliquot of the broth and placed it on the nutrient agar and subsequently performed the colony count. We replicated this test three times. Statistical analysis was performed using an ANOVA.

The biochemical properties of the colonies were determined by various tests; the tests were kept in anaerobic-aerobic conditions and observed 24 and 48 h after the bacteria were inoculated. Acid production from carbohydrates was assessed using API 20NE (BioMerieux, Inc.Durham, NC) supplemented by glucose, xylose, fructose and mannose tests. A drop of hydrogen peroxide was added to selected colonies for the catalase activity. Lecithinase, protease and lipolytic activity were evaluated using egg yolk agar, gelatin agar and tributyrin agar, respectively. All the species in this bacteria genus presents hemolytic activity; therefore, sheep blood agar was used in the biochemical characterization and identification of *P. luminescens*.

Genotypic characterization

Genomic DNA was extracted from the colonies isolated from *Photorhabdus* sp. SL0708 using the DNeasy Tissue Kit (Qiagen) following the manufacturer's protocol and stored in a 1.5 mL Eppendorf tube in TE buffer and RNase inhibitor at -20°C. We quantified the DNA by Nanodrop, using 100 to 300 ng for the PCR amplification, and agarose gel electrophoresis at 1%. For the partial amplification of the 16S rRNA sequence, we used primers 16SF (5´-GGA GAG TTA GAT CTT GGC TC-3´) and 16SR (5´-AAG GAG GTG ATC CAG CCG CA-3´) (Ruisheng and Grewal, 2010); these primers are commonly used for the identification of *Photorhabdus* sp. PCR conditions were as follows: denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min and a final period at 72°C for 10 min (Ehlers and Niemann, 1998). Sequencing of the resulting PCR products was carried out by SSIGMOL (Universidad Nacional de Colombia). The sequence generated by this study was deposited in GenBank [KC149508.1].

The sequence of the 16S rRNA region of *Photorhabdus* sp. SL0708 was assembled manually using BioEdit software. To identify the bacterial symbiont, we used, currently identified, 16S region sequences of *Photorhabdus* sp. as the *ingroup* (Table 1) and species of *Proteus vulgaris* Hauser (Enterobacter: Enterobacteriaceae) [AJ301683] and *Xenorhabdus nematophila* Thomas and Poinar (Enterobacter: Enterobacteriaceae) Breton strain [DQ282116.1] as outgroup. Using the second sequence structure, we used SINA (Silva server program) to perform the sequences multiple alignment. We then analyzed the alignment using the method of maximum likelihood (ML), inferring the appropriate substitution model of evolution by employing the BIC and AICc heuristic method and using Modeltest and Topali v2.5 programs. Lastly, we constructed a phylogenetic tree using PhyML by conducting a bootstrap analysis of 1000 replicates using the evolutionary model of substitution.

Pathogenicity evaluation

Using a 10 µl Hamilton syringe, 300 *G. mellonella* larvae were inoculated, behind the last pair of proleg, with 5 µl of 0, $1x10^2$, $1x10^4$ and $1x10^6$ cells/mL. Each concentration had five replicates and each experimental unit had 5 larvae. Mortality was assessed during 48 h; larvae color and bioluminescence were also verified.

Table 1. Sequences of 16s rRNA region of the different species of *Photorhabdus* sp used as in-group.

Specie	Strain	Genbank	Host Nematode	Author
P. asymbiotica asymbiotica	3265-8	NR_03685.1	Undefined	R. Akhurst
P.asymbiotica australis	9802892	NR_029093.1	Undefined	R. Akhurst
P. temperata temperata	XINACH	AJ007405	*H. megidis*	R. Akhurst
P. temperata khanii	Meg 1	AY278655	*H. megidis*	R. Akhurst
P. temperata khanii	Habana	EU930338	*Heterorhabditis* sp.	R. Akhurst
P. temperata tasmaniensis	T327	EU930339	*H. zealandica*	R. Akhurst
P. temperata tasmaniensis	NZH3	FJ844932.1	*H. zealandica*	R. Akhurst
P. temperata stackebrandtii	GPS11	EF467859.3	Undefined	An,R. Grewal,P.S.
P. luminescens akhurstii	FRG04	NR_028869.1	*H. indica*	Fischer-Le Saux
P. luminescens akhurstii	IS5	AY278645	Undefined	Marokhazi,J
P. luminescens caribbeanensis	HG26	FJ817460.1	*Heterorhabditis* sp.	H. Mauleón
P. luminescens caribbeanensis	HG29	EU930345.1	*H. bacteriophora*	H. Mauleón
P. luminescens hainanesis	C8404	FJ862004.1	*Heterorhabditis* sp.	R. Akhurst
P. luminescens kayaii	C8406	EU930343.1	*Heterorhabditis* sp.	R. Akhurst
P. luminescens kayaii	FR33	EU930333.1	Undefined	Domaine
P. luminescens kayaii	DSM15194	AJ560630.1	*H. bacteriophora*	Hazir,S.
P. luminescens kleinii	DMS23513	JX513408.1	*H. georgiana*	Pages,S
P. luminescens kleinii	KMD37	HM072284.1	*H. georgiana*	An,R. - Grewal,P.S
P. luminescens laumondii	TT01	NR_028870.1	*H. bacteriophora*	Fischer-Le Saux,M
P. luminescens laumondii	E21	EU930341.1	*H. bacteriophora*	G. Poinar
P. luminescens laumondii	HP88	AY278648.1	*H. bacteriophora*	Marokhazi,J.
P. luminescens luminescens	Hb	NR_037074.1	*H. bacteriophora*	R. Akhurst
P. luminescens luminescens	Hm	AY278640.1	*H. bacteriophora*	R. Akhurst
P. luminescens sonorensis	Carborca	JQ912644	*H. sonorensis*	Orozco,R.A
P. luminescens sonorensis	CH35	JQ912649	*H. sonorensis*	Orozco,R.A
P. luminescens thracensis	FR32	EU930335	Undefined	Domaine
P. luminescens thracensis	DSM15199		*Heterorhabditis* sp.	CIP

Similarly, a drop of hemolymph was extracted and plated on MacConkey, NBTA and nutrient agar to ensure their growth; we replicated this test three times. Statistical analysis was performed using an ANOVA and lineal regression. To establish concentration effect and determine the most effective concentration, we made multiple comparisons between treatments using a Tukey test ($p = 0.05$). The tests were performed using SPSS 21 software.

RESULTS AND DISCUSSION

Phenotypic characterization

Bacterial strain SL0708, isolated from nematode *Heterorhabditis* sp.SL0708, presented a Gram-negative bacillus morphology (Figure 1A), characteristic of species of *Photorhabdus* sp. (Boemare and Akhurst, 2006). At 72 h, the colonies were convex, slimy, shiny and small (1 mm in diameter). After 48 h of incubation in culture media, the colonies had different pigmentations, those in NBTA were dark blue-green (Figure 1B) attributed to the absorption of bromothymol blue and TTC reduction (Eun-Kyung et al., 2012; Orozco et al., 2013). The colonies in MacConkey took on the neutral red of the agar (Figure 1C), and those on the nutrient agar were shiny and yellow (Figure 1D). The production of diffusible pigments is characteristic of all species of *Photorhabdus* (Boemare and Arhurst, 2006); this is evident in colonies of SL0708 in TSA medium, which produced a dark brown pigment (Figure 1E). The colonies' optimum temperature for growth was 25°C, presenting the lowest growth at 30 and 35°C (Table 2). This agrees with reports of Akhurst and Boemare (1988), which state that the best growth temperature is 25°C. This bacterium, like all the species of *Photorhabdus,* is luminescent after 24 to 48 h post-incubation (Singh et al., 2012). The luminescence and morphological characteristics of the colonies confirm that the isolate is part of the genus *Photorhabdus*.

The biochemical assays showed that isolate SL0708 had properties similar to those described to date for *Photorhabdus* species (Table 3). Like all the species of *Photorhabdus*, this bacterium has a fermentative metabolism that uses xylose, glucose, mannose and fructose as carbohydrates sources, which demonstrates its facultative anaerobic capability (Waterfield et al., 2009). The colonies also induced a complete beta hemolysis on blood agar after 48 h of incubation (Weissfeld et al., 2005)

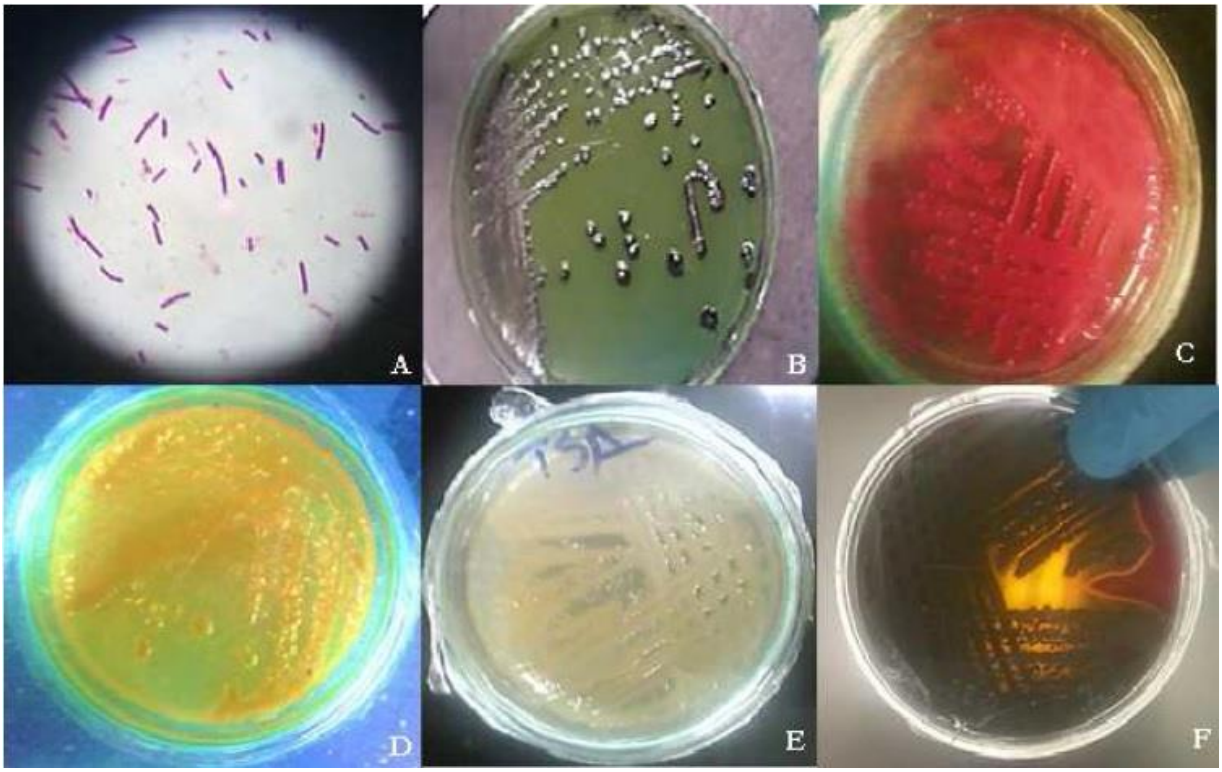

Figure 1. Growth of *P. luminescens* SL0708 in culture media and microscopy: A. Gram-negative bacilli. B. NBTA. C. MacConkey D. Nutrient medium. E. TSA. F. Blood agar.

Table 2. Optimum temperature for growth *P. luminescens* SL0708. *significant differences.

Temperature	cell/mL	F	p (<0.05)
25	$1x10^9$	135,967	0.0002*
30	$1x10^5$	101,876	0.4900
35	$1x10^4$	103,982	0.2000

(Figure 1F); this bacterium showed weak lecithinase and lipase activity, and no production of proteases, due to the absence of a protein hydrolysis halo on the gelatin agar. The weak activity of these enzymes is connected to the proliferation of phenotype phase II whose activities are negative or weak for these enzymes, as comparison to phase I (Burnell and Stock, 2000). Therefore, this isolate belongs to the species *P. luminescens.*

P. luminescens SL0708 differed from the other strain of *P. luminescens* by its ability to oxidize arabinose, that is, the microorganism has the necessary enzymes to oxidize and transform the monosaccharide into energy and use it as a carbon source. Because *P. luminescens* SL0708 is a facultative anaerobe, it can oxidize or ferment arabinose by using elements other than oxygen as terminal electron acceptors (Eun-Kyung et al., 2012). According to Singh et al. (2012), none of the species and subspecies of *Photorhabdus* have the ability to oxidize this monosaccharide. To date, *P. luminescens* SL0708 is the first species reported with this capability making this test (arabinose oxidation) an important indicator for the identification of these strain.

Genotypic characterization

The amplified 16S rRNA of strain SL0708 generated a 1500 pb band. Based on the comparison of the secondary structure of the 16S rRNA of other *Photorhabdus* species, the isolate SL0708 was classified as *P. luminescens* because of its 93% correspondence with the species of this group. The phylogenetic tree constructed in this study reveals that, *Photorhabdus* strain SL0708 is a species of the group *P. luminescens* and establishes *P. luminescens hainanesis* as a close group of these species (Figure 2). Tailliez et al. (2010) suggest this subspecies is a close group of *P. luminescens akhurstii*; however, based on this analysis, this subspecies is not a close group of *P. luminescens hainanesis* but a distant monophyletic group. Similarly, *P. sonorensis luminescens* is established as a close group of *P. luminescens luminescens*, *P. luminescens laumondii* as close group of *P. klenii luminescens* and distant of *P. luminescens*

Table 3. API 20NE reactions in six subspecies of *Photorhabdus* sp. different from *P. luminescens* SL0708.

Characteristic	*P. temperata. stackebrandtii*	*P. temperata temperata*	*P. luminescens akhurstii*	*P. luminescens laumondii*	*P. luminescens luminescens*	*P. luminescens kleinii*	*P. luminescens* SL0708
β Galactosidase	-	-	-	-	-	-	-
Arginine dihydrolase	+	-	-	-	-	-	-
Lisine descarboxylase	-	-	-	-	-	-	-
Ornithin descarboxylase	-	-	-	-	-	-	-
Citrate	-	+	+	-	+	-	+
H_2S	-	-	-	-	-	-	-
Urease	-	-	+	+	-	+	+
Tryptophan deaminase	-	-	-	-	-	-	-
Gelatin	+	+	+	+	+	+	+
Arabinose oxidation	-	-	-	-	-	-	+
Amygdalina oxidation	-	-	-	-	-	-	-
Glucose oxidation	+	+	+	+	+	+	+
Inositol oxidation	+	+	+	+	+	+	-
Mannitol oxidation	-	-	+	-	+	-	-
Melibiose oxidation	-	-	-	-	-	-	-
Rhamnose oxidation	-	-	-	-	-	-	-
Sucrose oxidation	-	-	-	-	-	-	-
Sorbitol oxidation	-	-	-	-	-	-	-

akhursti; this solves the polytomy presented by Orozco et al. (2013) between *P. luminescens sonorensis*, *P. luminescens laumondii* and *P. luminescens akhurstii*. The secondary structures of the nucleotide sequences are more evolutionarily conserved regions and have low nucleotide substitution (transversion) and low mutation rates (Vandamme, 2009). Using these secondary structures makes the analysis more accurate and valid, and their use probably allowed us to resolve the relationship of the three subspecies of *P. luminescens* correctly within the clade. Within the tree, *P. luminescens* and *P. temperata* are proposed as monophyletic groups while *P. asimbiotyca* is proposed as a possible paraphyletic group similar to that suggested by Tailliez et al. (2010). Orozco et al. (2013) reported on *P. asimbiotyca*, using a parsimony analysis, as a monophyletic group. The position of *P. asimbiotyca* within the genus *Photorhabdus* is still uncertain and varies according to the method of analysis.

Pathogenicity evaluation

Last instar larvae of *G. mellonella* were susceptible to *P. luminescens* SL0708, presenting a significant difference between treatments (F = 95.377; gl=3; p = 0.000; Figure 3) with a linear dependence between mortality and concentration. Tukey tests show that the concentration different was $1x10^6$ cells/ml. The corrected r^2 states that the independent variable concentration explains 90% of the behavior of the dependent variable mortality. Therefore the explanatory model is efficient. All dead larvae had the red coloration and bioluminescence typical of infection by *Photorhabdus* (Figure 4). We observed the highest mortality (>70%) after 24 h using a concentration of $1x10^6$ cells/mL at lower doses, larval mortality reduced and did not exceed 60%. This is similar to reports by Abdel-Razek (2002) and Forst and Nealson (1996), which state that a maximum concentration of $1x10^5$cel/mL mortality is 75% after 24 h. The virulence capability of the bacterial symbionts varies according to strains, cell concentration and the application method of the bacteria; the fastest method to bring about the death of the host is the intrahemolytic injection (Clarke and Dowds, 1995; Akhurst and Boemare, 1988). Moreover, *Photorhabdus* sp. bacteria have several virulence factors that directly affect the host's immune system contributing to its death. Recently, a major step characterized in insect immune response evasion, is the activity of the "make caterpillars floopy" (Mcf1) toxin and its effect on paralyzing hemocytes and the halting of their phagocytic capability, this seems to occur due to actin dynamics disruption (Lang et al., 2014). Among the virulence causing factors, there are enzymes such as metalloproteases, proteases, lipases and lecithinases, which degrade larva organelles causing irregularities in the body and eventual toxemia, and their ability to cause apoptosis in hosts' hemocytes and antiphagocytic activity, by affecting humeral and cellular responses (Waterfield et al., 2004). Other toxins that

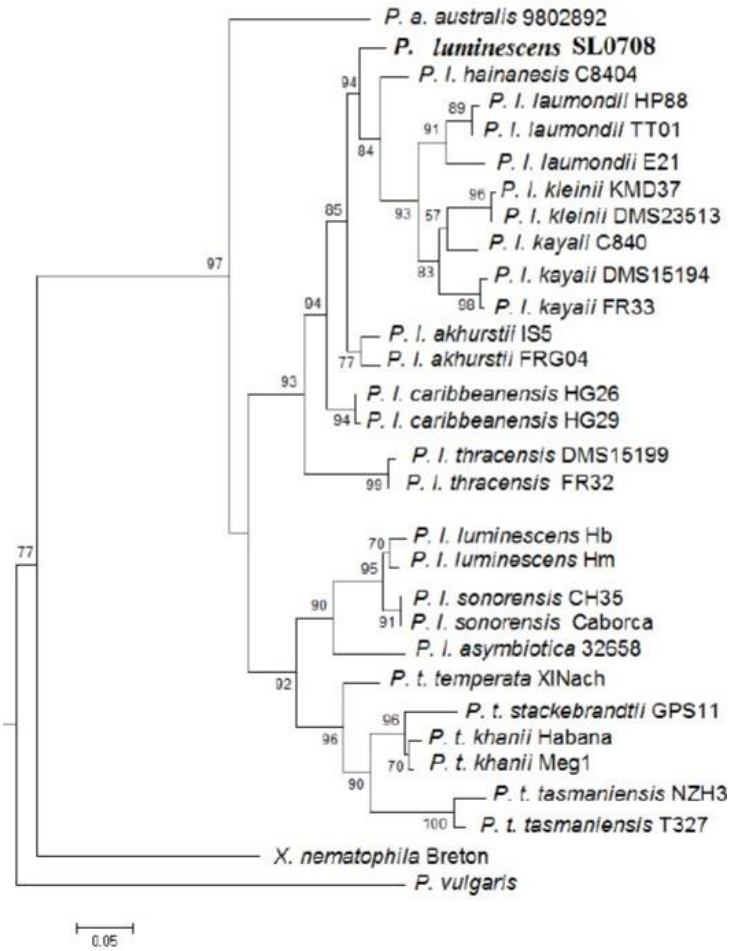

Figure 2. Maximum likelihood tree of 16S rRNA secondary structure, built under parameters HKY+I+G (Hasegawa et al., 1985). The numbers on the branches indicate bootstrap values of 1000 replicates. The tree was rooted with *P. vulgaris* and *X. nematophila*

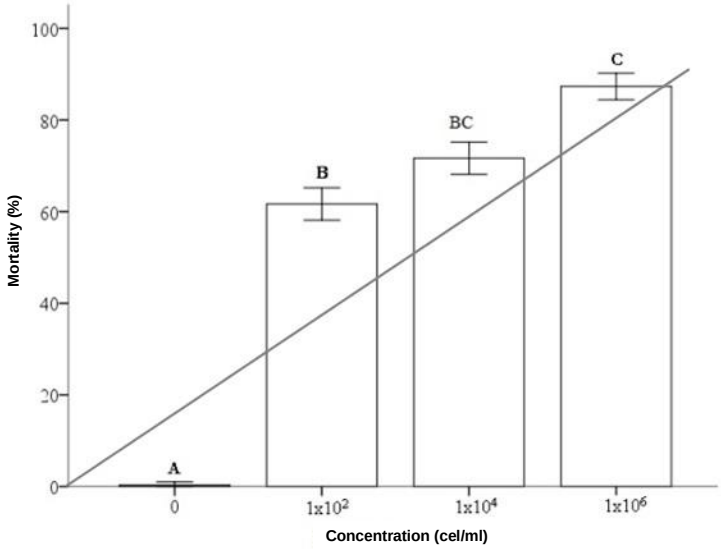

Figure 3. *G. mellonella* mortality (±SE) with four concentrations of *P. luminescens* SL0708. The letters above the bars show significant differences (P <0.005). The line shows lineal regression of value.

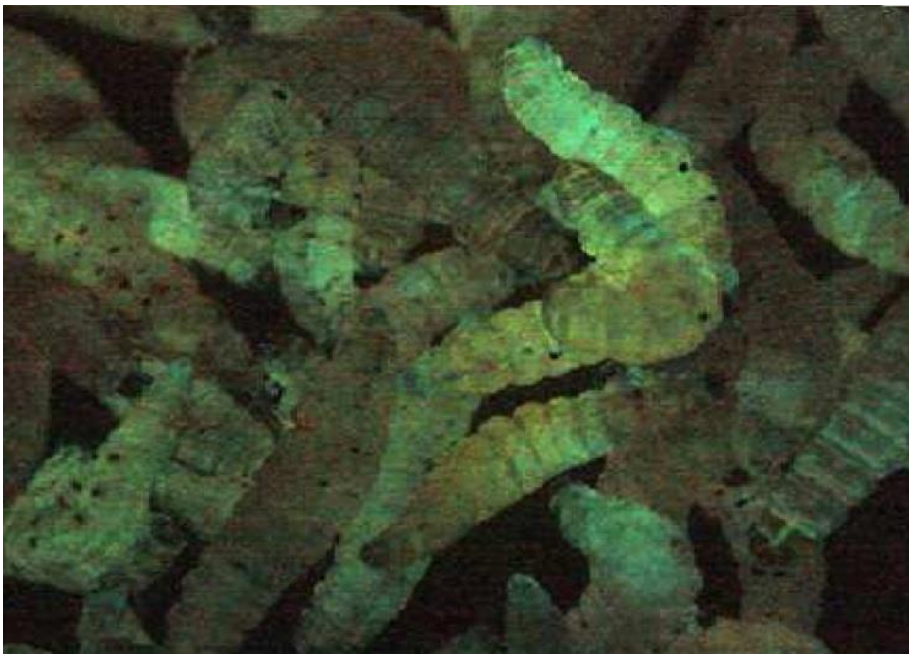

Figure 4. *P. luminescens* SL0708 bioluminescence in infected larvae after 24 h.

contribute towards insect lethality are the ones produced by the toxin complexes (Tcs), *Photorhabdus* insect related (Pir) proteins, and *Photorhabdus* virulence cassettes (PVCs). Several of the toxins produced by the bacterial symbiont protect the cadaver microenvironment due to their antimicrobial, antihelmintic and insecticidal activities (Orozco et al., 2013).

With the isolation and characterization of the bacterial symbiont *P. luminescens* SL0708, we can conclude that an approach to the characterization of the bacterial symbiont of the entomopathogenic nematode *Heterorhabditis* sp. SL0708 isolated in Valle del Cauca, Colombia, was completed. Also, we considered developing more detailed studies on this bacterial symbiont, as a potential source of natural products with applications not only in agriculture but also in the medical field, and develop *in vitro* production of nematode and to establish the interaction with its symbiont.

Conflict of Interests

The authors have not declared any conflict of interests.

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