African Journal of Biotechnology

Volume 13 Number 26, 25 June, 2014
ISSN 1684-5315

Academic Journals

ABOUT AJB

The **African Journal of Biotechnology (AJB)** (ISSN 1684-5315) is published weekly (one volume per year) by Academic Journals.

African Journal of Biotechnology (AJB), a new broad-based journal, is an open access journal that was founded on two key tenets: To publish the most exciting research in all areas of applied biochemistry, industrial microbiology, molecular biology, genomics and proteomics, food and agricultural technologies, and metabolic engineering. Secondly, to provide the most rapid turn-around time possible for reviewing and publishing, and to disseminate the articles freely for teaching and reference purposes. All articles published in AJB are peer-reviewed.

Submission of Manuscript

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

Click here to Submit manuscripts online

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

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

Editorial Board

Instructions for Author

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

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

Article Types
Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.
Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

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

Review Process

All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review.
Decisions will be made as rapidly as possible, and the journal strives to return reviewers' comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the AJFS to publish manuscripts within weeks after submission.

Regular articles

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

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

The Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length.. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.
Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

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

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

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

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

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

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

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

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

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

Examples:

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

Examples:

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

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

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

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

Short Communications

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

Fees and Charges: Authors are required to pay a $650 handling fee. Publication of an article in the African Journal of Biotechnology is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances

Submission of a manuscript implies: that the work described has not been published before (except in the form of an abstract or as part of a published lecture, or thesis) that it is not under consideration for publication elsewhere; that if and when the manuscript is accepted for publication, the authors agree to automatic transfer of the copyright to the publisher.

African Journal of Biotechnology

Table of Contents: Volume 13 Number 26, 25 June, 2014

ARTICLES

Table of Contents: Volume 13 Number 26, 25 June, 2014

academicJournals

Vol. 13(26), pp. 2593-2607, 25 June, 2014
DOI: 10.5897/AJB2013.13019
Article Number: 27F729B45604
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Review

Approaches to bioremediation of fossil fuel contaminated soil: An overview

Oghenekume G. Edeki and A. Keith Cowan*

Institute for Environmental Biotechnology, Rhodes University (EBRU), P. O. Box 94, Grahamstown 6140, South Africa.

Received 10 July, 2013; Accepted 10 June, 2014

A reliance on fossil fuels as a source of energy has resulted in the generation of pollutants which have entered the environment. Health of humans, animals, plants and microorganisms has been compromised due to activities linked to fossil fuel extraction, processing and use. Coal conversion to value added products has been investigated in an effort to reduce the cumulative effects of waste generated during mining. Clean coal technology, developed to convert coal into value added products with reduced pollution, has been a major source of liquid petroleum in South Africa. Although the conversion process, neither generates waste nor pollutes the soil environment, the final products either through accidental or deliberate spillage can have a severe and protracted impact. Biological methods for combating pollutants generated within the fossil fuels sector are preferred to mechanical or physicochemical practices. This is due to the production of non- or less toxic by-products, cost effectiveness and safety. In this manuscript, an overview of the approaches adopted and factors influencing microbial metabolism of fossil fuel contaminants in soil and water bodies is presented. In particular, emphasis is placed on bacteria as biocatalysts of choice and their ability to degrade waste coal and liquid petroleum hydrocarbons.

Key words: Fossil fuels, coal, petroleum hydrocarbons, biodegradation, pollutants.

INTRODUCTION

Fossil fuels are natural substances formed from the remains of ancient plants and animals. Over time, heat and pressure converted these remains into fuels which release energy when burned. The term fossil fuel also includes hydrocarbon-containing natural resources that are not derived from animal or plant sources. These are sometimes called mineral fuels. For the purpose of this review, the hydrocarbons derived from decayed plants and animals will be referred to as fossil fuels. The age of these ancient plant and animal fossil fuels is typically millions of years, and in some cases, in excess of 650 million years (Mann et al., 2003). Different types of fossil fuels are formed depending on the combination of animal and plant debris present. However, the length of time for which the material was buried and the temperature and pressure during decomposition also contributed to the

*Corresponding author. E-mail: a.cowan@ru.ac.za. Tel: +27 (0)46 6222 656. Fax: +27 (0)46 6222. 656.

type of fossil fuel formed. Fossil fuel has been broadly divided into three categories based on the mode of its formation. These are solid, liquid and gaseous fossil fuels and each is characterized by a high carbon and hydrogen content. Within these categories are volatile materials with low carbon: hydrogen ratios such as methane, liquid petroleum and the non-volatile materials composed almost of pure carbon, like anthracite coal. Fossil fuels have played an important role in providing energy for transportation, power generation, industrial growth, agricultural production and other basic human needs (Basha et al., 2009). Irrespective of the major roles that fossil fuels have played in sustaining the global economy, combustion of these fuels is a major source of anthropogenic CO_2 emissions (Muradov, 2001). For the purpose of this review, only the solid and liquid fossil fuels will be discussed.

Fossil fuel resources are generally a major source of revenue for the main oil and gas producing countries in Africa (Zalik and Watts, 2006). South Africa, which is one of the world's largest producers (5^{th}) and consumers (7^{th}) of fossil fuels (BP, 2012), has experienced a boost in her economy due to the production, consumption and exportation of coal (UNECA, 2011). Increased production over the last 30 years and an over reliance on coal as a source of energy has stimulated revenue accrual (ERC, 2004). BP statistics in 2011 showed that Africa has enormous potential in the fossil fuels sector with proven reserves accounting for about 9.5, 8 and 4% of the crude oil, natural gas and coal in the world, respectively (BP, 2011). The generation of electricity from fossil fuels cannot be neglected as more than 80% of electricity generated across the continent of Africa is from fossil fuels (IEA, 2011). The generation and supply of energy from fossil fuels has also been documented. IEA, in their 2011 annual report, stated that fossil fuels account for about 50% of the total energy supply and one-third of the energy consumed (IEA, 2011).

Huge problems have emerged due to an over reliance on fossil fuels and when viewed from an environmental and social perspective, it affects societies locally, regionally and globally (UNECA, 2011). Some of these problems include ozone depletion, global warming, acidification, and depletion of non-renewable resources. According to Höök and Tang (2013), energy production is the principal contributor to release of greenhouse gases, in particular CO_2, with fossil fuel combustion the major source. Of the three categories of fossil fuels, liquids (petroleum) and solids (coal) are the major contaminants in the environment. Any unwanted substance introduced into the environment is referred to as a 'contaminant' and the deleterious effects of these contaminants leads to 'pollution', a process in which a resource (natural or man-made) is rendered unfit for use, more often than not, by humans (Megharaj et al., 2011).

The drastic increase in the demand for coal has led to an increase in mining of this natural resource in countries like South Africa with subsequent generation of wastes and an increase in the level of pollution. One of the basic reasons behind the increase in demand for coal is due to the various products derived from coal during its conversion processes. For instance, in South Africa where liquid and gaseous fossil fuels are not readily available, coal liquefaction is one option available for obtaining these products. However, the utilization of coal and coal derived products is associated with serious environmental problems from the mining stage through to its final utilization by consumers (Geo-4, 2007). To reduce environmental damage by this energy source, new conversion technologies are urgently needed. One of the strategies adopted in reducing environmental damage is clean coal technology. Clean coal technologies which make use of biological processes to effect pollutant biodegradation have received considerable attention in recent years (Klein et al., 2008; Sekhohola et al., 2013). Similarly, the use of biocatalysts to remediate liquid petroleum hydrocarbon and diesel contaminated sites has been the subject of much recent attention (Sander et al., 2010; Diya'uddeen et al., 2011; Vaidehi and Kulkarni, 2012; Elazhari-Ali et al., 2013; Kang, 2014). In this paper, we present an overview of some of the approaches used in the biodegradation of coal, coal related contaminants, and liquid hydrocarbon pollutants in an effort to stimulate the search for and emergence of successful bioremediation strategies.

BIODEGRADATION OF COAL AND COAL-RELATED PRODUCTS

Studies on the breakdown of coal by bacteria and fungi started as far back as 1920 (Olson and Brinckman, 1986). Although, it was accepted that microorganisms are capable of degrading coal, significant research effort occurred only after demonstration of the successful breakdown of coal by bacteria (Fakoussa, 1981). One year later, Cohen and Gabriele (1982) demonstrated the breakdown of low rank leonardite using wood rot fungi. Following these breakthroughs, intensive study by various research groups was carried out with the aim of establishing a better understanding of the mechanisms involved in the biological transformation of coal and in combination with the production of value-added products (Polman et al., 1994; Fakoussa and Frost, 1999; Fakoussa and Hofrichter, 1999; Gotz and Fakoussa, 1999; Ralph and Catcheside, 1999; Machnikowska et al., 2002; Igbinigie et al., 2008; Jiang et al., 2013).

The complexity and recalcitrance of coal suggested initially that microorganisms might not be able to modify the physicochemical structure of this substrate. Thus, according to Klein et al. (2008), the colonization and breakdown of coal by microorganisms was not possible

unless certain necessary conditions such as moisture content, mineral salt availability, additional nitrogen sources and a stable pH were met. To date, a number of microorganisms have been identified as being able to modify the structure of coal (Yuan et al., 2006; Kang, 2014). Different mechanisms as suggested by various authors appear to be used to achieve modification of the coal structure and these include enzymatic changes (Cohen et al., 1987; Pyne et al., 1987; Fakoussa and Hofrichter, 1999), alkaline solubilisation (Strandberg and Lewis, 1987; Quigley et al., 1989a), metal ion chelation, and the action of surfactants (Fakoussa, 1988; Quigley et al., 1989b; Fredrickson et al., 1990).

Enzymatic modification of coal structure

A large number of biological molecules responsible for many chemical interconversions have been linked to the structural modification of coal otherwise called depolymerization (Hofrichter and Fakoussa, 2001). The depolymerization of brown coal occurs at low pH values (pH 3-6) resulting in the cleavage of bonds inside the coal molecular structure which leads to the formation of yellowish, fulvic-like substances with low molecular mass (Hofrichter and Fakoussa, 2001). Although a wide range of enzymes with coal degrading ability have been identified the majority appear to be from fungi. For instance, Sekhohola et al. (2013) provided a detailed list of the purported catalysts used in coal biodegradation which shows that nearly all of the enzymes that have been linked to coal biodegradation are of fungal origin. Even so, contradictory reports have been published with regard to fungal activity and breakdown of coal (Torzilli and Isbister, 1994). For instance, studies by Cohen et al. (1987) initially suggested that the ability of fungi to degrade coal was the result of enzymatic activity. However, in a subsequent report, these authors identified the coal solubilizing agent from *T. versicolor* by infrared spectroscopy and x-ray studies as ammonium oxalate monohydrate (Cohen et al., 1990) while Fredrickson et al. (1990) argued that the coal solubilizing activity of *T. versicolor* was not ammonium oxalate monohydrate but a siderophore-like compound. In addition to fungi, several gram positive and negative bacteria have been implicated in the biodegradation of coal.

Studies by Crawford and Gupta (1991) demonstrated that extracellular bacterial enzymes were capable of depolymerizing a soluble coal polymer although the enzymes involved were neither specified nor identified. Nevertheless, the depoly-merisation process appears to be non-oxidative which may indicate that non-oxidative, enzymatic depolymerization of coal is possible. Reports on the utilization of low rank coal as a source of carbon by several bacteria including *Pseudomonas oleovorans, Rhodococcus ruber* and *Bacillus* sp. Y7 have also been published (Fuchtenbusch and Steinbuchel, 1999; Jiang et al., 2013). The ability of *Bacillus* sp. Y7 to degrade lignite was attributed to the production of extracellular substances (Jiang et al., 2013) while oxidized lignin solubilisation was ~90% in the presence of *Pseudomonas putida* (Machnikowska et al., 2002). For the latter example however, it was stated that pre-treatment of lignite with nitric acid was essentially responsible for the enhanced rate of biodegradation. In an experiment carried out by Tripathi at al. (2010) on the fungal biosolubilisation of lignite and the subsequent production of humic acid, these authors concluded that the likely mechanism of lignin breakdown by fungi was somehow linked to action of oxidative (peroxidases and laccases) and hydrolytic enzymes (esterases) initially secreted by bacteria confirming an earlier observation based on a comparative study of coal solubilisation by both bacteria and fungi (Torzilli and Isbister, 1994). Some of the enzymes secreted by fungi which are believed to play a major role in the biodegradation of coal include lignin peroxidase (Hofrichter and Fritsche, 1997b; Laborda et al., 1999), laccase (Fakoussa and Frost, 1999), esterase (Laborda et al., 1999) and phenol oxidase (Laborda et al., 1999) and although their precise role in coal biodegradation remains unclear, a model for the phyto-biodegradation of low rank coal by mutualistic interaction between ligninolytic microorganisms and higher plants has recently been proposed (Sekhohola et al., 2013).

Alkaline substance modification of coal structure

A different mechanism of coal biodegradation has been suggested based upon results which indicate microbial secretion of alkaline substances that facilitate the breakdown of coal (Quigley et al., 1988). During this non-enzymatic process, often the formation of black liquids is observed coincident with higher pH (pH 7-10). The increase in pH has been attributed to the release of alkaline substances by bacteria which aid in coal solubilisation (Hofrichter and Fakoussa, 2001). The actual mechanism of coal biodegradation by bacteria due to alkaline substances is not well defined and as a consequence, not fully understood. Thus, Machnikowska et al. (2002), in an experiment on the microbial degradation of low rank coals, reported an increase in pH of medium containing sub-bituminous coal and suggested that the pH change arose as a result of the production of alkaline substances. Details of the alkaline substances involved however, in this and other studies and the effect of these on coal biodegradation remain obscure. As highlighted by Sekhohola et al. (2013) many different bacteria appear capable of secreting alkaline substances when inoculated into coal media including; *Pseudomonas putida, Arthrobacter* sp., *Streptomyces viridosporous, Streptomyces setonii, Bacillus pumilus,* and *Bacillus cereus.*

Metal ion chelation and the action of surfactants on the modification of coal

Experiments on coal bio-solubilisation carried out by Yin et al. (2011) pointed to the importance of surfactants in the synthesis of enzymes responsible for coal breakdown. These authors went further and showed that in the absence of surfactants; limited enzymes were adsorbed onto the coal surface while the reverse was the case in the presence of surfactants. Thus, interaction between enzyme and coal is possibly due to the presence of surfactants which modify the charge and the hydrophilic properties of the coal surface (Yin et al., 2011). Nonetheless, studies on the biological breakdown of coal have concentrated on fungi as the biocatalysts of choice and very few reports have examined the contribution by bacteria. A summary of the historical progress made so far in the field of coal biodegradation is presented in Table 1.

BIODEGRADATION OF PETROLEUM HYDROCARBONS

The biosolubilisation of coal and the serial production of liquid fuels has been investigated (Ackerson et al., 1990). In this report, bio-extracts from solubilized coal were converted to liquid alcohols, one of the earliest clean coal technologies for petroleum production. In South Africa many petroleum products are derived from coal using Fischer-Tropsch synthesis including fuels, plastics, oils, synthetic rubbers etc. Globally, there is high demand for petroleum products (Hasan et al., 2010) and during transportation of these from point of production to point of consumption spillage is inevitable (Das and Chandran, 2011). It has been estimated that natural crude oil seepage exceeds 600000 metric tons per year with a range of uncertainty of 200000 metric tons per year (Kvenvolden and Cooper, 2003). Accidental or deliberate release of crude oil into the environment has also led to serious pollution which affects both water and soil resources (Atlas, 1981; Okoh, 2006). Just like coal, different strategies including mechanical, chemical and biological have been developed and used to remediate sites contaminated with these petroleum hydrocarbons (Lohi et al., 2008).

A common mechanical means of remediating petroleum contaminated waters includes floating booms, skimmers, and oil-water separators (Ventikos et al., 2004; Yang et al., 2000). Unfortunately, removal of spilled oils from contaminated sites by these means is usually incomplete leading to progressive accumulation of residual hydrocarbons (Yang et al., 2000). Chemical remediation of oil contaminated sites on the other hand has been associated with increased dissolution of oil in seawater, which affects both water bodies and benthic biota (Doerffer, 1992). The reason why this technology is associated with increased dissolution of oil in water is because it makes use of chemical dispersants such as surfactants (Lohi et al., 2008). In contrast to the above, biological remediation technologies which have been intensively studied both in controlled conditions and field experiments (Okoh, 2006), appear to be the most environmentally friendly methods for removal of hydrocarbon pollutants (Barathi and Vasudevan, 2001; Balba et al., 2002; Urum et al., 2003; Liu et al., 2008; Das and Chandran, 2011). Bioremediation, which is one example of a biological remediation process, has been defined as the use of microorganisms to detoxify or remove pollutants from contaminated water and soil bodies (Medina-Bellver et al., 2005; Mukherjee and Bordoloi, 2012) and a comparison of treatment costs for South Africa reveals that it is by far the most economical technology (Table 2).

Different microorganisms including bacteria and fungi have been used to remediate hydrocarbon contaminated sites. Addition of nutrients to an oil spilled site to stimulate the growth of resident microorganisms in degrading contaminants is known as biostimulation while isolation, growth and introduction of microorganisms (that can degrade contaminants) from a different environment into oil spilled sites to remediate those sites is known as bioaugmentation. It has been argued that biostimulation is a superior technique to bioaugmentation (Alexander, 1999; Van Hamme et al., 2003; Philp and Atlas, 2005; Lohi et al., 2008) based on the outcome of field experiments (Abdulsalam et al., 2011). Studies by Devinny et al. (2000) and Bento et al. (2005) seem to support the above conjecture and show that augmented microorganisms easily lose their intrinsic degradation ability during the time it takes for acclimatisation to the new environment. Different amendments have been used to stimulate resident microorganism populations in oil spilled environments and a summary of these is presented in Table 3.

According to D'Annibale et al. (2006) and Yi et al. (2011), fungi are the organisms of choice with regards to bioaugmentation as these synthesize relatively unspecific enzymes involved in cellulose and lignin decay. Fungal enzymes degrade high molecular weight, complex and more recalcitrant toxic compounds, including aromatic structures (Grinhut et al., 2007; Mancera-Lopez et al., 2008). However, Sutherland (1992) explained how fungi degrade hydrocarbons indirectly by co-metabolism and stated that fungi generally do not utilize petroleum hydrocarbons (PHC) as their sole carbon and energy source but transform these compounds co-metabolically to detoxified metabolites. Different fungal species have been implicated in bio-augmentation studies involving both low and high molecular weight polyaromatic hydrocarbons (PAHs) in soils. For instance, Mancera-Lopez et al. (2008) carried out studies on petroleum hydrocarbon contaminated soils using *Rhizopus* sp.,

Table 1. Historical overview of advances in coal bioconversion.

Year	Progress	Reference
1981	Effects on hard coals by *Pseudomonas* strains, simultaneous biotenside-excretion	Fakoussa (1981)
1982	Solubilization of lignite to droplets on agar plates by fungal action	Cohen and Gabriele (1982)
1986	Acceleration of solubilisation by pre-treatment of coal	Scott (1986), Grethlein (1990)
1986	Solubilisation of coal by an extracellular component produced by *Streptomyces setonii* 75Vi2 in submerged culture	Strandberg and Lewis (1987)
1987	First solubilisation mechanism elucidated: production of alkaline substances (fungi + bacteria)	Quigley et al. (1988), Quigley et al. (1989a), Quigley et al. (1991)
1988	Second mechanism elucidated: production of chelating agents	Quigley et al. (1988), Quigley et al. (1989), Cohen et al. (1990), Quigley et al. (1991)
1989	First product on market: Solubilized lignite as fertilizer	Arctech Inc. (2007)
1991	Evidence that chelators alone are not responsible for all effects	Fakoussa (1994)
1994	Decolourisation and reduction of molecular weight of soluble lignite-derived humic acids proves catalytic enzymatic attack	Ralph and Catcheside (1994), Hofrichter and Fritsche (1997a and b)
1994	Analysis of low-molecular mass products from bio-solubilised coal	Toth-Allen et al. (1994)
1997	In vitro systems shown to degrade humic acids and attack matrix and coal particles	Hofrichter and Fritsche (1997a and b)
1997	First fine chemical produced successfully from heterogeneous humic acid mixtures to polyhydroxyalkanoates (PHA, “Bioplastic") by pure cultures	Fuchtenbusch and Steinbuchel (1999)
1999	Involvement of laccase in depolymerization of coal implied by conversion of coal humic acid to fulvic acids *in vivo* by *Trametes versicolor* (basidiomycetous fungi)	Fakoussa and Frost (1999)
2001	Microbial solubilisation of lignites. Preliminary gasification tests with solubilized coal yielding 21% energy recovery from methane	Gokcay et al. (2001)
2006	Mechanisms of coal solubilisation in *Penicillium decumbens* P6 combination of production of alkaline materials, peroxidase and esterase. First report on involvement of biosurfactant in coal solubilisation by fungi	Yuan et al. (2006)
2007	Degradation of LRC by *Trichoderma atrovide* (ES 11)	Silva-Stenico et al. (2007)
2007	Phytoremediation of coal mine spoil dump through integrated biotechnological approach	Juwarkar and Jambhulkar (2008)
2008	The effect of the particulate phase on coal biosolubilisation mediated by *Trichoderma atrovide* in a slurry bioreactor	Oboirien et al. (2008)
2008	Fungal biodegradation of hard coal by a newly reported isolate, *Neosartorya fischeri*	Igbinigie et al. (2008)

Table 1. Contd

2013	Formation of biosolubilised humic acid from lignite using *Bacillus* sp. Y7	Jiang et al. (2013)
2013	Production of methane from coal by a fungal isolate *Penicillium chrysogenum* MW1	Haider et al. (2013)

Table 2. A comparison of soil remediation treatment technology costs in South Africa.

Method of treatment	Approximate cost (ZAR/tonne soil)
Biological	70 - 2 395
Chemical	169 - 8 455
Physical	282 - 2 395
Solidification/stabilization	239 - 2 409
Thermal	423 - 10 569

Table 3. Examples of various biostimulation methods used to treat hydrocarbon contaminated sites.

Amendment type	Reference
Chelating agents	Da Silva et al. (2005)
Activated sludge from wastewater treatment	Juteau, et al. (2003), Maki et al. (1999)
Bio-solids and maize	Sarkar et al. (2005), Rivera-Espinoza and Dendooven (2004)
Immobilized-cell systems	Chen et al. (2009)
Nitrogen and phosphorous	Jiménez et al. (2006), Bento et al. (2005), Evans et al. (2004)
Surfactants or bio-surfactants	Rahman et al. (2002)
Bulking agents e.g. wheat straw, hay and sawdust	Namkoong et al. (2002), Rahman et al. (2002), Rhykerd et al. (1999)
Biocompatible hydrophobic solvents	Zawierucha et al. (2011)

Penicillium funiculosum and *Aspergillus sydowii* isolated from two aged soils contaminated with petroleum hydrocarbons and showed that each fungus was able to degrade PAHs effectively when compared to biostimulated soils. Bacteria on the other hand, though able to degrade aromatic hydrocarbons, only degrade low molecular weight PAHs. Many pure cultures of bacteria, including various strains of *Pseudomonas putida*, have been evaluated for their benzene, toluene and xylene (BTX) biodegradation potential (Jean et al., 2002, 2008). The highest PAHs that bacteria have been recorded to degrade are the PAHs containing four benzene rings such as pyrene and chrysene (Boonchan et al., 2000).

Mukherjee and Bordoloi (2011) reported that remediation of oil spilled sites usually requires the cooperation of more than a single species of microorganism because individual microorganisms can metabolize only a limited range of hydrocarbon substrates. Therefore, assemblages of mixed populations with overall broad enzymatic capabilities are required to energize the rate and extent of petroleum hydrocarbon degradation. Thus, various researchers have shown that consortia comprising bacteria and fungi are better bioaugmentation agents than individual bacterial and fungal isolates (Boonchan et al., 2000; Jacques et al., 2008). Table 4 presents a brief summary of single isolates of bacteria and fungi that are known to degrade aromatic hydrocarbons using bioaugmnetation as a strategy and various consortia of bacteria and fungi that successfully carry out this process.

Aliphatic hydrocarbons on the other hand which are basically made up of straight, branched and cyclic structures are more readily degraded by microorganisms than aromatic hydrocarbons (Das and Chandran, 2011). For instance, Colombo et al. (1996) investigated the biodegradation of aliphatic and aromatic hydrocarbons by natural soil microflora and pure cultures of imperfect and ligninolytic fungi. In their experiments, they discovered that the natural microbial soil assemblage isolated from an urban forest area was unable to significantly degrade

Table 4. A summary of microorganisms involved in the degradation of aromatic hydrocarbons using bioaugmentation as a strategy.

Microorganism	Contaminants treated	Reference
Single strains		
Mycobacterium sp.	Pyrene (PAH)	Heitkamp et al. (1988)
Pseudomonas paucimobilis	Fluoranthene (PAH)	Weissenfels et al. (1990)
Pseudomonas cepacia	HMW PAHs	Juhasz et al. (1996)
Sphingomonas paucimobilis	PAHs	Ye at al. (1996)
Burkholderia cepacia	fluoranthene, pyrene, benz[a]anthracene and dibenz[a,h]anthracene	Boonchan et al. (1998)
Comamonas testosteroni BR60	Crude oil, PAHs	Gentry et al. (2001)
Arthrobacter chlorophenolicus A6L	4-Chlorophenol	Jernberg and Jansso (2002)
Absidia cylindrospora	Fluorene	Garon et al. (2004)
Pseudomonas sp. ST41	Marine gas oil	Stallwood et al. (2005)
Pseudomonas aeruginosa WatG	Diesel oil	Ueno et al. (2006)
Sphingobium chlorophenolicum ATCC 39723	Pentachlorophenol	Dams et al. (2007)
Burkholderia sp. FDS-1	Fenitrothion	Hong et al. (2008)
Aspergillus sp. LEBM2	Phenol	Santos et al. (2008)
Gordonia sp. BS29	Aliphatic/aromatic hydrocarbons	Franzetti et al. (2009)
Pseudomonas putida ZWL73	4-Chloronitrobenzene	Niu et al. (2009)
Aspergillus sp.	LMW-PAHs (2–3 rings)	Silva et al. (2009a)
Trichocladium canadense, Fusarium oxysporum, Aspergillus sp.*, Verticillium* sp., *Achremonium* sp.	HMW-PAHs (4-7 rings)	Silva et al. (2009a)
Neosartorya sp. BL4	Total petroleum hydrocarbons	Yi et al. (2011)
Consortia		
Rhodococcus sp.*, Acinetobacter* sp., *Pseudomonas* sp.	PAHs (fluorene, phenanthrene, pyrene)	Yu et al. (2005)
Bacillus subtilis DM-04, *Pseudomonas aeruginosa* M and NM	Crude petroleum-oil hydrocarbons	Das and Mukherjee (2007)
Mycobacterium fortuitum, *Bacillus cereus*, *Microbacterium* sp., *Gordonia polyisoprenivorans, Microbacteriaceae bacterium*, *Fusarium oxysporum*	PAHs (anthracene, phenanthrene, pyrene)	Jacques et al. (2008)
Rhizopus sp., *Penicillium funiculosum*, *Aspergillus sydowii*	Petroleum hydrocarbons	Mancera-Lopez et al. (2008)
Bacillus strains B1F, B5A and B3G, *Chromobacterium* sp. 4015, *Enterobacter aglomerans* sp. B1A, *Achremonium* sp., *Aspergillus* sp., *Verticillium* sp.	Mixture of PAHs (naphthalene, phenanthrene, anthracene, pyrene, dibenzo[a]anthracene, benzo[a]pyrene)	Silva et al. (2009b)

crude oil, whereas pure fungi cultures effectively reduced the residues by 26-35% in 90 days. They also reported that normal alkanes were almost completely degraded in the first 15 days, whereas degradation of aromatic compounds (for example, phenanthrene and methylphenanthrene) exhibited slower kinetics. Another experiment conducted on the kinetics of the degradation of aliphatic hydrocarbons by the bacteria *Rhodococcus ruber* and *Rhodococcus erythropolis,* showed that the growth of these bacterial isolates on *n*-alkanes was intense when compared to growth in diesel medium (Zhukov et al., 2007). A comparative study on the degradation of both

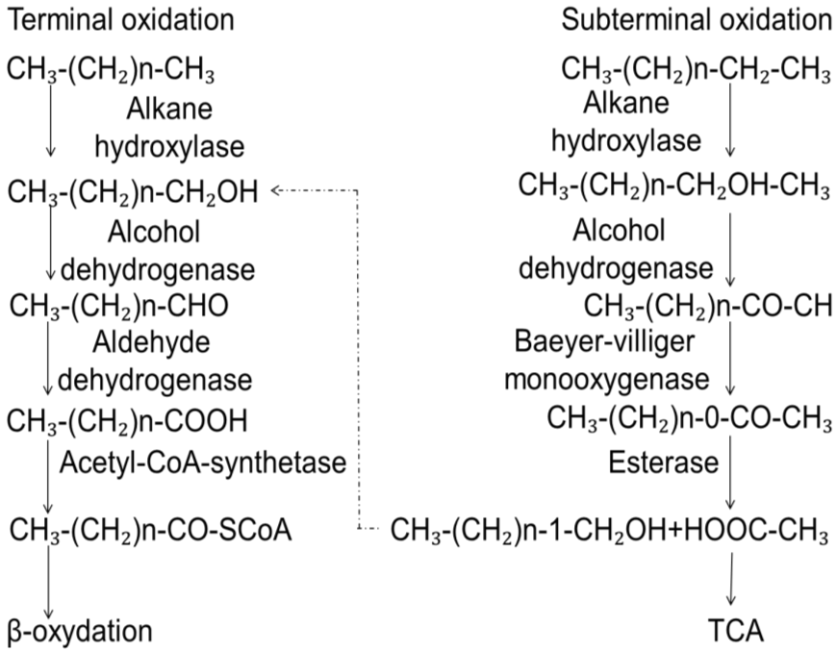


Figure 1. Simplified pathways for the degradation of *n*-alkanes by terminal and sub-terminal oxidation.

aliphatic and aromatic hydrocarbons by *Nocardia* sp. H17-1 was conducted and the results obtained showed a 99.0±0.1% and 23.8±0.8% reduction of both classes of hydrocarbons (Baek et al., 2006).

Mechanisms involved in petroleum hydrocarbon degradation

Various mechanisms of biodegradation of pollutants in the environment have been proposed by different researchers. For effective biodegradation of pollutants in environment, the chemicals must be accessible to the biological catalyst (Fritsche and Hofrichter, 2000). The first mechanism for degradation of petroleum hydrocarbons involves enzymes and was proposed by Fritsche and Hofrichter (2000). These authors stated that for complete degradation of the majority of organic pollutants to be accomplished, aerobic conditions are a requirement. Thus, introduction of oxygen into the environment is vital as a co-substrate in reactions catalysed by oxygenases and peroxidases (Kariga and Rao, 2011) which are the main enzymes responsible for the aerobic degradation of most pollutants. The conversion of organic pollutants step by step through peripheral pathways such as the tricarboxylic acid cycle into intermediates of central intermediary metabolism is one of the results achieved during the microbial degradation process (Fritsche and Hofrichter, 2000; Das and Chandran, 2011). Biosynthesis of cell biomass occurs from the central precursor metabolites acetyl-CoA, succinate, and pyruvate derived from sugars via gluconeogenesis. Different pathways for the aerobic degradation of the various components of petroleum hydrocarbons have been proposed. For instance *n*-alkanes, a major group in crude oil contamination have several pathways through which it is biodegraded.

Pathways for degradation of *n*-alkanes

Aerobic degradation of *n*-alkanes begins with the oxidation of a terminal methyl group which renders a primary alcohol to be oxidized to the corresponding aldehyde, and finally conversion into a fatty acid (van Hamme et al., 2003; Wentzel et al., 2007). The fatty acids which are formed are subsequently transformed to acyl-CoA by aldehyde dehydrogenase and acyl-CoA synthetase respectively (Wentzel et al., 2007). Figure 1 shows the general degradation pathways for *n*-alkanes by two types of oxidation systems. Different enzymes are involved in the initial terminal hydroxylation of *n*-alkanes by bacteria (van Beilen et al., 2003; van Beilen and Funhoff, 2007). Methane monooxygenases are the major group of enzymes that carry out the hydroxylation of short chain-length alkanes (C_2-C_4) (Hamamura et al., 1999) while the non-heme iron monooxygenases and soluble cytochrome P450 (CYP153) are known to degrade

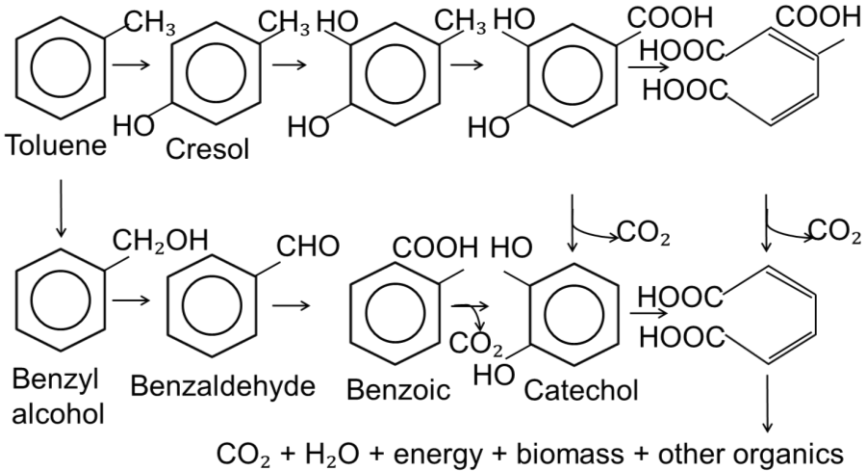


Figure 2. Mechanisms of toluene metabolism.

medium chain alkanes (C_5-C_{11}) (Maier et al., 2001; van Beilen et al., 2005). The long chain alkanes (C_{10}-C_{30}) are easily degraded by alkane hydroxylases such as LadA, the thermophilic flavin-dependent monooxygenase (Wentzel et al., 2007).

Anaerobic biodegradation of petroleum hydrocarbons has been achieved using different bacterial strains (Widdel et al., 2006; Foght, 2008; Salehi et al., 2008) and reports show that these bacteria activate hydrocarbons by unprecedented biochemical mechanisms that differ completely from those employed in aerobic hydrocarbon metabolism. These unprecedented biochemical mechanisms may be initiated by bacteria through metabolic pathways by oxygen-independent hydrocarbon-activating reactions (Heider and Schuhle, 2013).

Pathways for degradation of aromatic hydrocarbons

The biodegradation of aromatic compounds has been extensively studied due to its importance in the biogeochemical carbon cycle. Since many aromatic compounds such as benzene, toluene, ethylbenzene and xylene (BTEX) are major environmental pollutants; their detection and removal from contaminated sites are of great biotechnological interest (Dıaz et al., 2013). Different catabolic pathways for the degradation of aromatic compounds have been described. According to Harayama et al. (1999), toluene is degraded by bacteria along five different pathways and for the purposes of this review, only two of these pathways are highlighted.

Different enzymes are involved in the degradation of toluene and they include toluene monooxygenase, benzyl alcohol dehydrogenase, benzaldehyde dehydrogenase and catechol-2, 3-dioxygenase. These enzymes are organized into two different pathways with the upper pathway, coding for enzymes that convert aromatic alcohols to acids, while the lower pathway is involved in aromatic acid metabolism via an *ortho* and *meta* pathway (Hamzah et al., 2011). Ring hydroxylation, which is involved in the second pathway, yields methyl catechol as the metabolic intermediate with toluene dioxygenase as the key enzyme. A second mechanism involved in petroleum hydrocarbon degradation involves attachment of microbes to the substrates while a third mechanism involves the production of bio-surfactants (Figure 2).

Factors influencing rate of petroleum hydrocarbon biodegradation

For a successful bioremediation technology to be achieved, a full knowledge of the characteristics of the contaminated site and the parameters that affect the biodegradation of the pollutants must be accounted for. Different abiotic factors have been highlighted in various studies which influence the rate of hydrocarbon degradation in any environment. These factors include temperature, pH, nutrient availability, moisture content, and chemical composition of the contaminant, salinity of the environment, concentration and physical state of the contaminant (Leahy and Colwell, 1990; Salleh et al., 2003; Okoh, 2006).

The effect of temperature on the degradation of pollutants is very important as it affects solubility of the contaminants in the environment (Foght et al., 1996). Degradation of hydrocarbons occurs over a very wide range of temperatures.

However, the biodegradability of a contaminant decreases with a decrease in temperature (Das and Chandran, 2011). Researchers have isolated a number of hydrocarbon utilizing bacteria which include psychrotrophic, mesophilic and thermophilic bacteria. Psychrotrophic bacteria such as *Rhodococcus* sp. were reported

by Whyte et al. (1998 and 1999) to have successfully degraded short chain alkanes at 0°C. However in a report by Atlas (1981), a direct correlation between increased microbial degradation with an increase in temperature was recorded. This means that when microorganisms that are isolated from a cold region are introduced into an environment that has an elevated temperature, their metabolic activities tend to be faster (Atlas, 1981). According to Okoh (2006), highest degradation rates generally occur in the range 30-40°C in soil environments, 20-30°C in some fresh water environments, and 15-20°C in marine environments.

Biodegradation rates have also been measured in relation to pH (Strandberg and Lewis, 1988). Outcomes from various experiments conducted show that biodegradation is effectively carried out at an optimum of pH 7.0 (Zaidi and Imam, 1999). In a contaminated environment such as soil that is acidic in nature, the dominant microbial species that are capable of metabolising the contaminants in a short space of time appear to be fungi (Jones et al., 1970). The isolation of bacteria from an alkaline medium that were able to degrade phenol at pH 7.0-10.6 has also been reported (Kanekar at al., 1999). The importance of nutrients in the degradation of hydrocarbons has also been stressed (Cooney, 1984). During biodegradation of hydrocarbons, lack of nutrients such as nitrogen, phosphorus, potassium, and iron may either hinder the breakdown process or result in an incomplete breakdown of contaminants. In fresh water environments, nutrients are particularly deficient. The supply of carbon significantly increases during major oil spills in marine and fresh waters with nitrogen and phosphorus serving as limiting factors (Atlas, 1985). A deficiency in these nutrients in fresh water is due to demand by plants, and photosynthetic and non-photosynthetic microorganisms. Enhancement of biodegradation in different experiments has been achieved through the addition of nutrient supplements (Breedveld and Sparrevik, 2000; Li et al., 2006; Xia et al., 2006; Vyas and Dave, 2010). It should be noted however, that excessive nutrient concentration can impact the microbial degradation of hydrocarbons negatively (Oudot et a., 1998; Chaîneau et al., 2005).

The stability of water activity in aquatic environments has caused researchers to focus more attention on soils. For instance, Bossert and Bartha (1984) stated that the water activity of an aquatic environment is 0.98 while that of soil has a range between 0.0 and 0.99. The wide range of water activity in soils has made biodegradation of petroleum hydrocarbons very difficult. For effective biodegradation in soils, water activity must be kept constant and at an optimum level.

The chemical composition of contaminants in any environment is another factor that influences microbial degradation of such contaminants. Petroleum hydrocarbons which is made up of four classes; saturates, aromatics, asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and resins, differ in their susceptibility to microbial attack. Biodegradation of hydrocarbons in decreasing order of susceptibility is ranked in the following order: *n*-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes (Leahy and Colwell, 1990). According to Okoh (2006) the biodegradation of heavier crude oils is generally much more difficult than lighter ones. However, a report contrary to that of Okoh (2006) published by Cooney et al. (1985) stated that the degradation of more complex compounds such as naphthalene was faster than that of hexadecane in water-sediment mixtures from a freshwater lake. This observed result could be as a result of the action of co-metabolism by the organisms acting on the substrates.

Metabolic rate of microorganisms in mineralising contaminants in different environments tends to decline with increasing salinity (Ward and Brock, 1978; Minai-Tehrani et al., 2006). The ability of different microorganisms to degrade hydrocarbons in contaminated environments in the presence of elevated concentration of salts has been tested. Results showed that almost 100% of initial phenanthrene and dibenzothiophene were degraded at a salt concentration of 35 g/L (Díaz et al., 2002) while Abed et al. (2006) reported that at salinities ranging from 60 to 140 g/L, alkane biodegradation rates were 50 to 60% with a lesser degradation rate of less than 30% at 180 g/L. Contrary to these reports, Bertrand et al. (1990) isolated an Achaeon from a water-sediment interface with salinity of 310 g/L which was able to degrade eicosane more efficiently at a rate of 64% in a medium that contained sodium chloride at a concentration of 146 g/L.

Due to the dispersion of oil in water during spillage, a slick typically forms which gives rise to emulsions (mousse) (Leahy and Colwell, 1990). The formation of an emulsion in water increases the surface tension of the oil thereby making it available for microorganisms to degrade (Salleh et al., 2003). Emulsion formation through microbial production and release of biosurfactants has been documented (Kosaric, 2001; Kumar et al., 2008; Aparna et al., 2011; Mnif et al., 2011). Kumar et al. (2008) reported that a hydrocarbon degrading and biosurfactant producing strain of *Pseudomonas*, DHT2, which was isolated from oil contaminated soil was able to degrade crude oil, fuels, alkanes and PAHs. These authors also established that the biosurfactants which were produced by the organism lowered the surface tension of the medium from 54.9 to 30.2 dN/cm and formed a stable emulsion.

CONCLUSION

Bacterial degradation of fossil fuels (solids and liquids) is an important and emerging aspect of biotechnology which

is neither fully described nor understood and as a consequence, technologies for implementation as commercial remediation strategies are few. While fungal biodegradation/biosolubilisation of coal and coal related products has been widely reported, it appears that work with bacteria has lagged and in some cases it has been completely ignored. In contrast, the use of bacteria and bacterial consortia for the remediation of petroleum hydrocarbon contamination is well established (Pinedo-Rivilla et al., 2009; Basha et al., 2010; Zhang et al., 2013; Ma et al., 2013; Martin et al., 2013) and as a consequence, commercial remediation protocols and the associated biocatalysts are widely available. Even so, there is a growing realisation that a mutualistic relationship between microorganisms and higher plants is necessary for complete remediation of contaminated sites (Ndimele, 2010; Sekhohola et al., 2013). Thus, further study is needed to enhance our understanding of the processes involved in the bacterial bioconversion of coal and petroleum hydrocarbon contaminants in order to facilitate both a reduction in pollutant levels and to explore the potential for generating products of value. While the use of single strains to degrade coal and liquid hydrocarbon contaminants has been widely reported, consortia of bacteria or bacteria together with fungi appear to be the biocatalysts of choice as biodegradation agents.

Conflict of Interests

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

ACKNOWLEDGMENTS

The authors are grateful for financial support provided by Anglo American Thermal Coal and Coastal and Environmental Services (CES). Mr. Oghenekume G. Edeki acknowledges financial support from Anglo American Thermal Coal and the National Research Foundation (IFR1202220169, Grant No: 80879) in the form of a doctoral bursary.

REFERENCES

Abdulsalam S, Bugaje IM, Adefila SS, Ibrahim S (2011). Comparison of biostimulation and bioaugmentation for remediation of soil contaminated with spent motor oil. Int. J. Environ. Sci. Technol. 8:187-194.

Abed RMM, Al-Thukair A, De Beer D (2006). Bacterial diversity of a cyanobacterial mat degrading petroleum compounds at elevated salinities and temperatures. FEMS Microbiol. Ecol. 57:290-301.

Ackerson MD, Johnson NL, Le M, Clausen EC, Gaddy JL (1990). Biosolubilization and liquid fuel production from coal. Appl. Biochem. Biotechnol. 24/25:913-928.

Alexander M (1999). Biodegradation and bioremediation. Academic Press, San Diego, CA, USA.

Aparna A, Srinikethan G, Hegde S (2011). Effect of addition of biosurfactant produced by *Pseudomonas sps*. on biodegradation of crude oil. 2011 2nd International Conference on Environmental Science and Technology. IACSIT Press, Singapore.

Arctech Inc. (2007). Balancing the earth with pre-combustion carbon sequestration. http://www.arctech.com/micgas.html

Atlas RM (1981). Microbial degradation of petroleum hydrocarbons:an environmental perspective. Microbiol. Rev. 45:180-209.

Atlas RM (1985). Effects of hydrocarbons on micro-organisms and biodegradation in arctic ecosystems. In:Engelhardt FR, editor. Petroleum Effects in the Arctic Environment. London, UK. pp. 63–99.

Baek K, Yoon B, Oh H, Kim H, Lee I (2006). Biodegradation of aliphatic and aromatic hydrocarbons by *Nocardia* sp. H17-1. Geomicrobiol. J. 23(5):253-259.

Balba MT, Al-Shayji Y, Al-Awadhi N, Yateem A (2002). Isolation and characterization of biosurfactant producing bacteria from oil contaminated soil. Soil Sed. Contam. 11:41-55.

Barathi S, Vasudevan N (2001). Utilization of petroleum hydrocarbons by *Pseudomonas fluorescens* isolated from petroleum contaminated soil. Environ. Int. 26:413-416.

Basha KM, Rajendran A, Thangavelu V (2010). Recent advances in the biodegradation of phenol:a review. Asian J. Exp. Biol. Sci. 1:219-234.

Basha S, Gopal K, Jebaraj S (2009). A review on biodiesel production, combustion, emissions and performance. Renew. Sustain. Environ. Rev. 13:1628-1634.

Bento FM, Camargo AO, Okeke BC, Frankenberger WT (2005). Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation, and bioaugmentation. Bioresour. Technol. 96:1049-1055.

Bertrand JC, Al-Mallah M, Acquaviva M, Mille G (1990). Biodegradation of hydrocarbons by an extremely halophilic archaebacterium. Lett. Appl. Microbiol. 11:260-263.

Boonchan S, Britz ML, Stanley GA (1998). Surfactant-enhanced biodegradation of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophilia*. Biotechnol. Bioeng. 59:482-494.

Boonchan S, Britz ML, Stanley GA (2000). Degradation and mineralization of high molecular-weight polycyclic aromatic hydrocarbons by defined fungal–bacterial co-cultures. Appl. Environ. Microbiol. 66:1007-1019.

Bossert I, Bartha R (1984). The fate of petroleum in soil ecosystems in:*Petroleum Microbiology*. Macmillan Publishing Co., New York, USA.

BP (2011). BP Statistical Review of World Energy. http://www.google.co.za/url?sa=t&rct=j&q=&esrc=s&source=web&cd=14&cad=rja&uact=8&ved=0CEUQFjADOAo&url=http%3A%2F%2Fbakerinstitute.org%2Ffiles%2F1596%2F&ei=1jB6U7SfNMnvOsWxgbAH&usg=AFQjCNFPe-qKlPOBt7ngnNApYVuH5hRV-Q&sig2=r5ufb92hYzqvtOc4AGCM1A

BP (2012). BP Statistical Review of World Energy June 2012. http://www.google.co.za/url?sa=t&rct=j&q=&esrc=s&source=web&cd=2&cad=rja&uact=8&ved=0CDMQFjAB&url=http%3A%2F%2Fwww.bp.com%2Fcontent%2Fdam%2Fbp%2Fpdf%2FStatistical-Review-2012%2Fstatistical_review_of_world_energy_2012.pdf&ei=5i56U9usOszEPZvlgdgG&usg=AFQjCNFHKtmbHFvnFfNTw-xPnJkDnL5VsQ&sig2=C0czyvld7jZPIGnMZCNCFg

Breedveld GD, Sparrevik M (2000). Nutrient-limited biodegradation of PAH in various soil strata at a creosote contaminated site. Bioedegradation 11:391-399.

Chaîneau CH, Rougeux G, Yéprémian C, Oudot J (2005). Effects of nutrient concentration on the biodegradation of crude oil and associated microbial populations in the soil. Soil Biol. Biochem. 37:1490-1497.

Chen B, Yen C, Hsueh C (2009). Cost-effective biostimulation strategy for waste water decolorization using immobilized-cell systems. Bioresour. Technol. 100:2975-2981.

Cohen M, Feldman KA, Brown CS, Gray ET (1990.) Isolation and identification of the coal-solubilizing agent produced by *Trametes versicolor*. Appl. Environ. Microbiol. 56:3285-3291.

Cohen MS, Bowers WC, Aronson H, Gray ET (1987). Cell-free

solubilization of coal by *Polyporus versicolor.* Appl. Environ. Microbiol. 53:2840-2843.
Cohen MS, Gabriele PD (1982). Degradation of coal by the fungi *Polyporus versicolor* and *Poria monticola*. Appl. Environ. Microbiol. 44:23-27.
Colombo JC, Cabello M, Arambarri AM (1996). Biodegradation of aliphatic and aromatic hydrocarbons by natural soil microflora and pure cultures of imperfect and lignolitic fungi. Environ. Pollut. 94:355-362.
Cooney JJ (1984). The fate of petroleum pollutants in fresh water ecosystems in:Petroleum microbiology. Macmillan, New York, NY, USA.
Cooney JJ, Silver SA, Beck EA (1985). Factors influencing hydrocarbon degradation in three freshwater lakes. Microb. Ecol. 11:127-137.
Crawford DL, Gupta RK (1991). Characterization of extracellular bacterial enzymes which depolymerize a soluble lignite coal polymer. Fuel 70:577–580.
D'Annibale A, Rosetto F, Leonardi V, Federici F, Petruccioli M (2006). Role of autochthonous filamentous fungi in bioremediation of a soil historically contaminated with aromatic hydrocarbons. Appl. Environ. Microbiol. 72:28-36.
Da Silva MLB, Ruiz-Aguilar GML, Alvarez PJJ (2005). Enhanced anaerobic biodegradation of BTEX-ethanol mixtures in aquifer columns amended with sulphate, chelated ferric iron or nitrate. Biodegradation 16:105-114.
Dams RI, Paton G, Killham K (2007). Bioaugmentation of pentachlorophenol in soil and hydroponic system. Int. Biodeterior. Biodegrad. 60:171–177.
Das K, Mukherjee AK (2007). Crude petroleum-oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India. Biores. Technol. 98:1339-1345.
Das N, Chandran P (2011). Microbial degradation of petroleum hydrocarbon contaminants:an overview. Biotechnol. Res. Int. 1:1-13.
Devinny J, Chang SH (2000). Bioaugmentation for soil bioremediation in:Bioremediation of contaminated soils. Marcel Dekker, New York, USA. pp. 466-488.
Dıaz E, Jiménez JI, Nogales J (2013). Aerobic degradation of aromatic compounds. Curr. Opin. Biotechnol. 24:431-442.
Díaz MP, Boyd KG, Grigson SGW, Burgess JG (2002). Biodegradation of crude oil across a wide range of salinities by an extremely halotolerant bacterial consortium MPD-M, immobilized onto polypropylene fibers. Biotechnol. Bioeng. 79:145-153.
Diya'uddeen BH, Wan Daud WMA, Abdul Aziz AR (2011). Treatment technologies for petroleum refinery effluents: A review. Proc. Safe. Environ. Prot. 89:95-105.
Doerffer JW (1992). Chemical response technology to an oil spill in: Oil spill response in the marine environment. Pergamon Press, Oxford, UK.
Elazhari-Ali A, Singh AK, Davenport RJ, Head IM, David Werner (2013). Biofuel components change the ecology of bacterial volatile petroleum hydrocarbon degradation in aerobic sandy soil. Environ. Pol. 173:125-132.
ERC (2004). Energy for sustainable development: South African Profile, Phase 1 Final Report. http://www.google.co.za/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&cad=rja&uact=8&ved=0CCwQFjAA&url=http%3A%2F%2Fwww.erc.uct.ac.za%2FResearch%2Fpublications%2F04ERC-Energy%2520for%2520sustainable%2520development%2520phase1.pdf&ei=LzF6U9XLIInIPLnwgJgL&usg=AFQjCNFtiNyWMNoTsPtWPnCT5t9B4KB8bQ&sig2=ClnFbzzQAt6YEUrYRoAV-A
Evans FF, Rosado AS, Sebastián GV, Casella R, Machado PLOA, Holmström C (2004). Impact of oil contamination and biostimulation on the diversity of indigenous bacterial communities in soil microcosms. FEMS Microbiol. Ecol. 49:295-305.
Fakoussa RM (1981). Coal as a substrate for microorganisms. Investigations of the microbial decomposition of (untreated) bituminous coals. Ph.D. Dissertation, Rhein Friedrich-Wilhelm University, Germany.
Fakoussa RM (1988). Production of water-soluble coal-substances by partial microbial liquefaction of untreated hard coal. Resour. Conserv. Recycl. 1:251-260.
Fakoussa RM (1994). The influence of different chelators on the solubilization/liquefaction of different pretreated and natural lignites. Fuel Proc. Technol. 40:183-192.
Fakoussa RM, Frost PJ (1999). In vivo-decolorization of coal derived humic acids by laccase-excreting fungus *Trametes versicolor*. Appl. Microbiol. Biotechnol. 52:60-65.
Fakoussa RM, Hofrichter M (1999). Biotechnology and microbiology of coal degradation. Appl. Microbiol. Biotechnol. 52:25-40.
Foght J (2008). Anaerobic biodegradation of aromatic hydrocarbons:Pathways and prospects. J. Mol. Microbiol. Biotechnol. 15:93-120.
Foght JM, Westlake DWS, Johnson WM, Ridgway HF (1996). Environmental gasoline-utilizing isolates and clinical isolates of *Pseudomonas aeruginosa* are taxonomically indistinguishable by chemotaxonomic and molecular techniques. Microbiology 142:2333-2340.
Franzetti A, Caredda P, Ruggeri C, La Colla L, Tamburini E, Papacchini M (2009). Potential application of surface active compounds by *Gordonia* sp. strain BS29 in soil remediation technologies. Chemosphere 75:801-807.
Fredrickson JK, Stewart DL, Campbell JA, Powell MA, McMullock M, Pyne JW, Bean RM (1990). Biosolubilization of low-rank coal by a *Trametes versicolor* siderophore-like product and other complexing agents. J. Ind. Microbiol. 5:401-406.
Fritsche W, Hofrichter M (2000). Aerobic degradation by microorganisms: principles of bacterial degradation in:Biotechnology, Environmental Processes II, Vol. IIb. Wiley-VCH, Weinhein, Germany.
Fuchtenbusch B, Steinbuchel A (1999). Biosynthesis of polyhydroxyalkanoates from low rank coal liquefaction products by *Pseudomonas oleovorans* and *Rhodococcus ruber*. Appl. Microbiol. Biotechnol. 52:91-95.
Garon D, Sage L, Wouessidjewe D, Seigle-Murandi F (2004). Enhanced degradation of fluorine in soil slurry by *Absidia cylindrospora* and maltosyl-cyclodextrin. Chemosphere 56:159-66.
Gentry TJ, Newby DT, Josephson KL, Pepper IL (2001). Soil microbial population dynamics following bioaugmentation with a 3-chlorobenzoate-degrading bacterial culture. Biodegradation 349:349-357.
Global Environmental Outlook-4 (Geo-4)(2007). Environment for Development. United Nations Environmental Programme.
Gokcay CF, Kolankaya N, Dilek FB (2001). Microbial solubilization of lignites. Fuel 80:1421-1433.
Gotz GKE, Fakoussa RM (1999). Fungal biosolubilization of Rhenish brown coal monitored by Curie point pyrolysis/gas chromatography/mass spectrometry using tetraethylammonium hydroxide. Appl. Microbiol. Biotechnol. 52:41-48.
Grethlein HE (1990). Pretreatment of Lignite in: Bioprocessing and biotreatment of coal. Dekker, New York.
Grinhut T, Hadar Y, Chen Y (2007). Degradation and transformation of humic substances by saprotrophic fungi: processes and mechanisms. Fungal Biol. Rev. 21:179-189.
Haider R, Ghauri MA, SanFilipo JR, Jones EJ, Orem,WH, Tatu CA, Akhtar K, Akhtar N (2013). Fungal degradation of coal as a pretreatment for methane production. Fuel 104:717-725.
Hamamura N, Storfa RT, Semprini L, Arp DJ (1999). Diversity in butane monooxygenases among butane-grown bacteria. Appl. Environ. Microbiol. 65:4586-4593.
Hamzah A, Tavakoli A, Rabu A (2011). Detection of toluene degradation in bacteria isolated from oil contaminated soils. *Sains Malaysiana* 40:1231-1235.
Hasan SW, Ghannam MT, Esmail N (2010). Heavy crude oil viscosity reduction and rheology for pipeline transportation. Fuel 89:1095-1100.
Heider J, Schuhle K (2013). Anaerobic biodegradation of hydrocarbons including methane in the prokaryotes: Prokaryotic physiology and biochemistry. 4th Edition. Springer, Heidelberg, New York, Dordrecht, London.

Heitkamp MA, Freeman JP, Miller DW, Cerniglia CE (1988). Pyrene-degradation by a *Mycobacterium sp*.: identification of oxidation and ring fission products. Appl. Environ. Microbiol. 54:2556-2565.
Hofrichter M, Fakoussa R (2001). Microbial Degradation and Modification of Coal in:Lignin, humic substances and coal. Wiley-VCH, Weinheim, Germany.
Hofrichter M, Fritsche W (1997a). Depolymerization of low-rank coal by extracellular fungal enzyme systems. II. The ligninolytic enzymes of the coal-humic-acid-depolymerizing fungus *Nematoloma frowardii* b19. Appl. Microbiol. Biotechnol. 47:419-424.
Hofrichter M, Fritsche W (1997b). Depolymerization of low-rank coal by extracellular fungal enzyme systems. III. In vitro depolymerization of coal humic acids by a crude preparation of manganese peroxidase from the white-rot fungus *Nematoloma frowardii* b19. Appl. Microbiol. Biotechnol. 47:566-571.
Hong Q, Zhang Z, Hong Y, Li S (2008). A microcosm study on bioremediation of fenitrothion-contaminated soil using *Burkholderia sp. FDS-1*. Int. Biodeterior. Biodegrad. 59:55-61.
Höök M, Tang X (2013). Depletion of fossil fuels and anthropogenic climate change - A review. Energy Policy 52:797-809.
Igbinigie EE, Aktins S, van Breugel Y, van Dyke S, Davies-Coleman MT, Rose PD (2008). Fungal biodegradation of hard coal by a newly reported isolate, *Neosartorya fischeri.* Biotechnol. J. 3:1407-1416.
International Energy Agency (IEA) (2011). Key world energy statistics. http://www.google.co.za/url?sa=t&rct=j&q=&esrc=s&source=web&cd=2&cad=rja&uact=8&ved=0CDQQFjAB&url=http%3A%2F%2Fwww.iea.org%2Fpublications%2Ffreepublications%2Fpublication%2Fkey_world_energy_stats-1.pdf&ei=CDJ6U7mQEcOCOMXzgegI&usg=AFQjCNGTQS30fgycpBRBLLzZ6ZRo9M5FBA&sig2=CGnxOYwSo-85GTPqNxihOg.
Jacques RJS, Okeke BC, Bento FM, Teixeira AS, Peralba MCR, Comargo FAO (2008). Microbial consortium bioaugmentation of a polycyclic aromatic hydrocarbons contaminated soil. Bioresour. Technol. 99:2637-2643.
Jean JS, Tsai CL, Ju SH, Tsao CW, Wang SM (2002). Biodegradation and transport of benzene, toluene, and xylenes in a simulated aquifer: comparison of modelled and experimental results. Hydrol. Proc. 16:3151-3168.
Jean JS, Wang Lee MK, Chattopadhyay SM, Maity JP (2008). Effects of inorganic nutrient levels on the biodegradation of benzene, toluene, and xylene (BTX) by *Pseudomonas* spp. in a laboratory porous media sand aquifer model. Bioresour. Technol. 99:7807-7815.
Jernberg C, Jansson JK (2002). Impact of 4-chlorophenol contamination and/or inoculation withthe4-chlorophenol-degrading strain, *Arthrobacter chlorophenolicus* A6L, on soil bacterial community structure. FEMS Microbiol. Ecol. 42:387-397.
Jiang F, Li Z, Lv Z, Goa T, Yang J, Qin Z, Yuan H (2013). The biosolubilization of lignite by Bacillus sp. Y7 and characterization of the soluble products. Fuel 103:639-643.
Jiménez N, Vinas M, Sabaté J, Díez S, Bayona JM, Solanas AM, Albaiges J (2006). The Prestige oil spill. 2. Enhanced biodegradation of a heavy fuel oil under field conditions by the use of an oleophilic fertilizer. Environ. Sci. Technol. 40:2578-2585.
Jones JG, Knight M, Byron JA (1970). Effect of gross pollution by kerosene hydrocarbons on the microflora of moorland soil. Nature 227:1166.
Juhasz AL, Britz ML, Stanley GA (1996). Degradation of high molecular weight polycyclic aromatic hydrocarbons by *Pseudomonas cepacia*. Biotechnol. Lett. 18:577-582.
Juteau P, Bisaillon J, Lépine F, Ratheau V, Beaudet R, Villemu R (2003). Improving the biotreatment of hydrocarbons-contaminated soils by addition of activated sludge taken from the wastewater treatment facilities of an oil refinery. Biodegradation 14:31-40.
Juwarkar AA, Jambhulkar HP (2008). Phytoremediation of coal mine spoil dump through integrated biotechnological approach. Bioresour. Technol. 99:4732-4741.
Kanekar PP, Sarmaik SS, Kelkar AS (1999). Bioremediation of phenol by alkaliphilic bacteria isolated from alkaline lake of Lonar, India. J. Appl. Microbiol. 1:128-133.
Kang JW (2014). Removing environmental organic pollutants with bioremediation and phytoremediation. Biotechnol. Lett. 36(6):1129-39.
Karigar CS, Rao SS (2011). Role of microbial enzymes in the bioremediation of pollutants: A review. Enzyme Res. 2011:1-11.
Klein J, Fakoussa R, Holker U, Hofrichter R, Schmiers H, Sinder C, Steinbuchel A (2008). Biotechnology of Coal in: Biotechnology. Special Processes, Wiley VCH, Weinheim, Germany.
Kosaric N (2001). Biosurfactants and their application for soil bioremediation. Food Technol. Biotechnol. 39:295-304.
Kumar M, León V, Materano AD, Ilzins OA, Luis L (2008). Biosurfactant production and hydrocarbon-degradation by halotolerant and thermotolerant *Pseudomonas sp.* World J. Microbiol. Biotechnol. 24:1047-1057.
Kvenvolden KA, Cooper CK. (2003). Natural seepage of crude oil into the marine environment. Geo-Mar. Lett. 23:140-146.
Laborda F, Monistrol IF, Luna N, Fernández M (1999). Processes of liquefaction/solubilization of Spanish coals by microorganisms. Appl. Microbiol. Biotechnol. 52:49-56.
Leahy JG, Colwell RR (1990). Microbial degradation of hydrocarbons in the environment. Microbiol. Rev. 54:305-315.
Li Y, Lei Z, Zhang Z, Sugiura N (2006). Effects of nutrient addition on phenol biodegradation rate in biofilm reactors for hypersaline wastewater treatment. Environ. Technol. 27:511-520.
Liu PG, Whang L, Yang M, Cheng S (2008). Biodegradation of diesel-contaminated soil: A soil column study. J. Chin. Inst. Chem. Eng. 39:419-428.
Lohi A, Cuenca AM, Anania G, Upreti SR, Wan L (2008). Biodegradation of diesel fuel-contaminated wastewater using a three-phase fluidized bed reactor. J. Hazard. Mater. 154:105-111.
Ma F, Shi S-N, Sun T-H, Li A. Zhou J-T, Qu Y-Y (2013). Biotransformation of benzene and toluene to catechols by phenol hydroxylase from *Arthrobacter* sp. W1. Appl. Microbiol. Biotechnol. 97:5097-5103.
Machnikowska H, Pawelec K, Podgórska A (2002). Microbial degradation of low rank coals. Fuel Proc. Technol. 77:17-23.
Maier T, Forster HH, Asperger O, Hahn U (2001) Molecular characterization of the 56-kDa CYP153 from *Acinetobacter* sp. EB104. Biochem. Biophys. Res. Commun. 286:652-658.
Maki H, Sasaki T, Sasaki E, Ishihara M, Goto M, Harayama S (1999). Use of wastewater sludge for the amendment of crude oil bioremediation in meso-scale beach simulating tanks. Environ. Technol. 20:625-632.
Mancera-Lopez ME, Esparza-Garcıa F, Chavez-Gomez B, Rodrıguez-Vazquez R, Saucedo-Castaneda G, Barrera-Cortes J (2008). Bioremediation of an aged hydrocarbon- contaminated soil by a combined system of biostimulation-bioaugmentation with filamentous fungi. Int. Biodeterior. Biodegrad. 61:151-60.
Mann P, Gahagan L, Gordon MB (2003). Tectonic setting of the world's giant oil and gas fields in:Giant oil and gas fields of the decade, 1990-1999. Amer. Assoc. Petrol. Geol.Tulsa Okla. USA.
Martin F, Malagnoux L, Violet F, Jakoncic J, Jouanneau Y (2013). Diversity and catalytic potential of PAH-specific ring-hydroxylating dioxygenases from a hydrocarbon-contaminated soil. Appl. Microbiol. Biotechnol. 97:5125-5135.
Medina-Bellver JI, Marín P, Delgado A, Rodríguez-Sánchez A, Reyes E, Ramos JL Marqués S (2005). Evidence for *in situ* crude oil biodegradation after the prestige oil spill. Environ. Microbiol. 7:773-779.
Megharaj M, Ramakrishnan B, Venkateswarlu K, Sethunathan N Naidu R (2011). Bioremediation approaches for organic pollutants: A critical perspective. Environ. Int. 37:1362-1375.
Minai-Tehrani D, Herfatmanesh A, Azari-Dehkordi F, Minuoi S (2006). Effect of salinity on biodegradation of aliphatic fractions of crude oil in soil. Pak. J. Biol. Sci. 9:1531-1535.
Mnif S, Chamkha M, Labat M, Sayadi S (2011). Simultaneous hydrocarbon biodegradation and biosurfactant production by oilfield-selected bacteria. J. Appl. Microbiol. 111:525-536.
Mukherjee AK, Bordoloi NK (2011). Bioremediation and reclamation of soil contaminated with petroleum oil hydrocarbons by exogenously

seeded bacterial consortium:a pilot-scale study. Environ. Sci. Pollut. Res. 18:471-478.
Mukherjee AK, Bordoloi NK (2012). Biodegradation of benzene, toluene, and xylene (BTX) in liquid culture and in soil by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains and a formulated bacterial consortium. Environ. Sci. Pollut. Res. 19:3380-3388.
Muradov N (2001). Hydrogen via methane decomposition:an application for decarbonization of fossil fuels. Int. J. Hydrogen Energy 26:1165-1175.
Namkoong W, Hwang EY, Park JS, Choi JY (2002). Bioremediation of diesel-contaminated soil with composting. Environ. Pollut. 119:23-31.
Ndimele PE (2010). A review on the phytoremediation of petroleum hydrocarbon. Pak. J. Biol. Sci. 13:715-722.
Niu G-L, Zhang J-J, Zhao S, Liu H, Boon N, Zhou N-Y (2009). Bioaugmentation of a 4-chloronitrobenzene contaminated soil with *Pseudomonas putida ZWL73*. Environ. Pollut. 157:763-771.
Oboirien BO, Burton SG, Cowan D, Harrison STL (2008). The effect of the particulate phase on coal biosolubilisation mediated by *Trichoderma atroviride* in a slurry bioreactor. Fuel Proc. Technol. 89:123-130.
Okoh AI (2006). Biodegradation alternative in the clean-up of petroleum hydrocarbon pollutants. Biotechnol. Mol. Biol. Rev. 138-50.
Olson GJ, Brinckman FE (1986). Bioprocessing of coal. Fuel 65:1638-1646.
Oudot J, Merlin FX, Pinvidic P (1998). Weathering rates of oil components in a bioremediation experiment in estuarine sediments. Mar. Environ. Res. 45:113-125.
Philp JC, Atlas RM (2005). Bioremediation of contaminated soils and aquifers. ASM Press, Washington DC, USA.
Pinedo-Rivilla C, Aleu J, Collado IG (2009). Pollutants biodegradation by fungi. Curr. Org. Chem.13:1194-1214.
Polman JK, Stoner DL, Delezene-Briggs KM (1994). Bioconversion of coal, lignin and dimethoxybenzyl alcohol by *Penicillium citrinum*. J. Ind. Microbiol. 13:292-299.
Pyne JW, Stewart DL, Fredrickson J, Wilson BW (1987). Solubilization of leonardite by an extracellular fraction from *Coriolus versicolor.* Appl. Environ. Microbiol. 53:2844-2848.
Quigley DR, Breckenridge CR, Dugan PR (1989b). Effects of multivalent cations on low-rank coal solubilities in alkaline solutions and microbial cultures. Energy Fuels 3:571-574.
Quigley DR, Breckenridge CR, Polman JK, Dugan PR (1991). Hydrogen peroxide, peroxidase and low rank coal. Fuel 70:581-583.
Quigley DR, Ward B, Crawford DL, Hatcher HJ, Dugan PR (1989a). Evidence that microbially produced alkaline materials are involved in coal solubilization. Appl. Biochem. Biotechnol. 20/21:753-763.
Quigley DR, Wey JE, Breckenridge CR, Stoner DL (1988). The influence of pH on biological solubulization of oxidized, low-rank coal. Res. Conserv. Recyc. 1:163-174.
Rahman KSM, Banat IM, Thahira J, Thayumanavan T, Lakshmanaperumalsamy P (2002). Bioremediation of gasoline contaminated soil by a bacterial consortium amended with poultry litter, coir pith and rhamnolipid biosurfactant. Bioresour. Technol. 81:25-32.
Ralph JP, Catcheside DEA (1994). Decolourisation and depolymerisation of solubilised low-rank coal by the white-rot basidiomycete *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. 42:536-542.
Ralph JP, Catcheside DEA (1999). Transformation of macromolecules from a brown-coal by lignin peroxidase. Appl. Microbiol. Biotechnol. 52:70-77.
Rhykerd RL, Crews B, McInnes KJ, Weaver RW (1999). Impact of bulking agents, forced aeration, and tillage on remediation of oil-contaminated soil. Bioresour. Technol. 67:279-285.
Rivera-Espinoza Y, Dendooven L (2004). Dynamics of carbon, nitrogen and hydrocarbons in diesel-contaminated soil amended with biosolids and maize. Chemosphere 54:379-386.
Salehi R, Shayegan J, Ghavipanjeh F, Pazouki M, Hosseinnia A (2008). Anaerobic bioconversion of heavy hydrocarbons using native consortia. Iran J. Chem Eng. 6:40-49.
Salleh AB, Ghazali FM, Abd Rahman RNZ, Basri M (2003). Bioremediation of petroleum hydrocarbon pollution. Ind. J. Biotechnol. 2:411-425.
Sander AB, Weelink SAB, van Eekert MHA, Stams AJM (2010). Degradation of BTEX by anaerobic bacteria: physiology and application. Rev. Environ. Sci. Biotechnol. 9:359-385.
Santos EO, Rosa CFC, Passos CT, Sanzo AVL, Burkert JFM, Kalil SJ, Burkert CAV (2008). Pre-screening of filamentous fungi isolated from a contaminated site in Southern Brazil for bioaugmentation purposes. Afr. J. Biotechnol. 7:1314-1317.
Sarkar D, Ferguson M, Datta R, Birnbaum S (2005). Bioremediation of petroleum hydrocarbons in contaminated soils: Comparison of biosolids addition, carbon supplementation, and monitored natural attenuation. Environ. Pollut. 136:187-195.
Sekhohola LM, Igbinigie EE, Cowan AK (2013). Biological degradation and solubilisation of coal. Biodegradation 24:305-318.
Silva IS, dos Santos EC, de Menezes CR, de Faria AF, Franciscon E, Grossman M (2009b). Bioremediation of a polyaromatic hydrocarbon contaminated soil by native soil microbiota and bioaugmentation with isolated microbial consortia. Bioresour. Technol. 100:4669-4675.
Silva IS, Grossman M. Durrant LR (2009a). Degradation of polycyclic aromatic hydrocarbons (2–7 rings) under micro aerobic and very-low-oxygen conditions by soil fungi. Int. Biodeterior. Biodegrad. 63:224-229.
Silva-Stenico ME, Vengadajellum CJ, Janjua HA, Harrison STL, Burton SG, Cowan DA (2007). Degradation of low rank coal by *Trichoderma atroviride* ES11. J. Ind. Microbiol. Biotechnol. 34:625-631.
Stallwood B, Shears J, Williams PA, Hughes KA (2005). Low temperature bioremediation of oil-contaminated soil using biostimulation and bioaugmentation with a *Pseudomonas sp.* from maritime Antarctica. J. Appl. Microbiol. 99:794-802.
Strandberg GW, Lewis SN (1987). Solubilization of coal by an extracellular product from *Streptomyces setonii* 75Vi2. J. Ind. Microbiol. 1:371-375.
Strandberg GW, Lewis SN (1988). Factors affecting coal solubilization by the Bacterium *Streptomyces setonii* 75Vi2 and by alkaline buffers. Appl. Biochem. Biotechnol. 18:355-361.
Sutherland JB (1992). Detoxification of polycyclic aromatic hydrocarbons by fungi. J. Ind. Microbiol. 9:53-62.
Torzilli AP, Isbister JD (1994). Comparison of coal solubilisation by bacteria and fungi. Biodegradation 5:55-62.
Toth-Allen J, Torzilli AP, Isbister JD (1994). Analysis of low-molecular mass products from biosolubilized coal. FEMS Microbiol. Lett. 116:283-286.
Tripathi RC, Jain VK, Tripathi PSM (2010). Fungal biosolubilization of Neyveli lignite into humic acid. Energy Sources Part A Recover. Util. Environ. Effects 32:72-82.
Ueno A, Hasanuzzaman M, Yumoyo I, Okuyama H (2006). Verification of degradation of n-alkanes in diesel oil by *Pseudomonas aeruginosa* strain WatG in soil microcosms. Curr. Microbiol. 52:182-185.
UNECA (2011). Fossil fuels in Africa in the context of a carbon constrained future. http://www.google.co.za/url?sa=t&rct=j&q=&esrc=s&source=web&cd=2&cad=rja&uact=8&ved=0CDQQFjAB&url=http%3A%2F%2Fwww.uneca.org%2Fsites%2Fdefault%2Ffiles%2Fpublications%2Fwp12-fossil_fuels_in_africa.pdf&ei=5jJ6U8XKCcbsOuKwgeAK&usg=AFQjCNGmcARgpq8qlH4hUJOJtlpCTM5glw&sig2=m_BVoWXPbrLSCPx-NQhoyw.
Urum K, Pekdemir T, Gopur M (2003). Optimum conditions for washing of crude oil-contaminated soil with biosurfactant solutions. Process safety and environmental protection. Trans. Inst. Chem. Eng. 81:203-209.
Vaidehi K, Kulkarni SD (2012). Microbial remediation of polycyclic aromatic hydrocarbons: An overview. Res. J. Chem. Environ.16:200-212.
van Beilen JB, Funhoff EG (2007). Alkane hydroxylases involved in microbial alkane degradation. Appl. Microbiol. Biotechnol. 74:13-21.
van Beilen JB, Li Z, Duetz WA, Smits THM, Witholt B (2003). Diversity of alkane hydroxylase systems in the environment. Oil Gas Sci. Technol. 58:427-440.
van Beilen JB, Smits TH, Roos FF, Brunner T, Balada S B,

Rothlisberger M, Witholt B (2005). Identification of an amino acid position that determines the substrate range of integral membrane alkane hydroxylases. J. Bacteriol. 187:85-91.
van Hamme JD, Singh A, Ward OP (2003). Recent advances in petroleum microbiology. Microbiol. Mol. Biol. Rev. 67:503-549.
Ventikos NP, Vergetis E, Psaraftis EN, Triantafyllou G (2004). A high-level synthesis of oil spill response equipment and countermeasures. J. Hazard. Mater. 107:51-58.
Vyas TK, Dave BP (2010). Effect of addition of nitrogen, phosphorus and potassium fertilizers on biodegradation of crude oil by marine bacteria. Ind. J. Mar. Sci. 39:143-150.
Ward DM, Brock TD (1978). Hydrocarbon biodegradation in hypersaline environments. Appl. Environ. Microbiol. 35:353-359.
Weissenfels WD, Beyer M, Klein J (1990). Degradation of fluoranthene by pure bacterial cultures. Appl. Microbiol. Biotechnol. 32:479-484.
Wentzel A, Ellingsen TE, Kotlar HK, Zotchev SB, Throne-Holst M (2007). Bacterial metabolism of long-chain n-alkanes. Appl. Microbiol. Biotechnol. 76:1209-1221.
Whyte LG, Hawari J, Zhou E, Bourbonniére L, Inniss WE, Greer CW (1998). Biodegradation of variable chain length alkanes at low temperature by a psychrotrophic *Rhodococcus* sp. Appl. Environ. Microbiol. 64:2578-2584.
Whyte LG, Slagman SJ, Pietrantonio F, Bourbonniére L, Koval SF, Lawrence JR, Inniss WE Greer CW (1999). Physiological adaptations involved in alkane assimilation at a low temperature by *Rhodococcus sp.* Strain Q15. Appl. Environ. Microbiol. 65:2961-2968.
Ye D, Siddiqi MA, Maccubbin AE, Kumar S, Sikka HC (1996). Degradation of polynuclear aromatic hydrocarbons by *Sphingomonas paucimobilis*. Environ. Sci. Technol. 30:136-142.
Yi T, Lee E, Park H, Cho K (2011). Biodegradation of petroleum hydrocarbons by *Neosartorya* sp. BL4. J. Environ. Sci. Health Part A. 46:1763-1768.
Yin S, Tao X, Shi K (2011). The role of surfactants in coal bio-solubilisation. Fuel Process Technol. 92:1554-1559.
Widdel F, Boetius A, Rabus R (2006). Anaerobic biodegradation of hydrocarbons including methane. Prokaryotes 2:1028-1049.
Xia WX, Li JC, Zheng XL, Bi XJ, Shao JL (2006). Enhanced biodegradation of diesel oil in seawater supplemented with nutrients. Eng. Life Sci. 6:80-85.
Yang LM, Lai C, Shieh WK (2000). Biodegradation of dispersed diesel fuel under high salinity conditions. Water Res. 34:3303-3314.
Yu KS, Wong AH, Yau KW, Wong YS, Tam NF (2005). Natural attenuation, biostimulation and bioaugmentation on biodegradation of polycyclic aromatic hydrocarbons (PAHs) in mangrove sediments. Mar. Pollut. Bull. 51:1071-1077.
Yuan H, Yang J, Chen W (2006). Production of alkaline materials, surfactants and enzymes by *Penicillium decumbens* strain P6 in association with lignite degradation/solubilisation. Fuel 85:1378-1382.
Zaidi BR, Imam SH (1999). Factors affecting microbial degradation of polycyclic aromatic hydrocarbon phenanthrene in the Caribbean coastal water. Mar. Pollut. Bull. 38:737-742.
Zalik A, Watts M (2006). Imperial oil: Petroleum politics in the Nigerian Delta and the new scramble for Africa. Soc. Rev. 2005.
Zawierucha I, Malina G, Singh A, Parmar N, Kuhad RC (2011). Bioremediation of contaminated soils:effects of bioaugmentation and biostimulation on enhancing biodegradation of oil hydrocarbons. Springer, Berlin, Heidelberg, Germany.
Zhang W, Yin K, Chen L (2013). Bacteria-mediated bisphenol A degradation. Appl. Microbiol. Biotechnol. 97:5681-5689.
Zhukov DV, Murygina VP, Kaliuzhnyi SV (2007). Kinetics of the degradation of aliphatic hydrocarbons by the bacteria *Rhodococcus ruber* and *Rhodococcus erythropolis*. Prikl. Biokhim. Mikrobiol. 43:657-663.

academicJournals

Vol. 13(26), pp. 2608-2612, 25 June, 2014
DOI: 10.5897/AJB2014.13816
Article Number: D9E864E45605
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Molecular marker screening of peanut (*Arachis hypogaea* L.) germplasm for *Meloidogyne arenaria* resistance

Valéria Carpentieri-Pípolo[1]*, Maria Gallo-Meagher[2], Don W. Dickson[3], Daniel W. Gorbet[2], Maria De Lurdes Mendes[4] and Silvia Graciele Hülse de Souza[5]

[1]Agronomy Department, Universidade Estadual de Londrina, CP 6001, Londrina, PR, 86051-990, Brazil.
[2]University of Florida, IFAS, PO Box 110300, Gainesville, FL, 32611-0300, USA.
[3]University of Florida, IFAS - 2212 Building 970, PO Box 110620, Gainesville, FL ,32611-0620, USA.
[4]University of Florida, IFAS - 2212 Building 970, PO Box 110620, Gainesville, FL, 32611-0620, USA.
[5]Laboratory of Molecular Biology, Universidade Paranaense, CP224, Umuarama, 87502-210, Brazil.

Received 25 March, 2014; Accepted 2 June, 2014

A restriction fragment length polymorphism (RFLP) marker linked to a locus for resistance to *Meloidogyne arenaria* (Neal) Chitwood race 1, along with visual evaluation following root staining were used to screen four breeding populations and three lines of peanut (*Arachis hypogaea* L.) in a root-knot nematode infested field. COAN and Florunner peanut cultivars were used as resistant and susceptible controls, respectively. Genomic DNA was isolated from young leaves of plants during the growing season, and Southern blot analysis was conducted using RFLP probe R2430E. Only COAN and the line TP301-1-8 were homozygous for the resistance marker. During evaluation, root masses were counted and the resistance phenotype scored. This field data confirmed the RFLP marker results. Except for COAN and TP301-1-8, all other genotypes displayed high levels of nematode reproduction. The RFLP probe R2430E provided a useful marker for identifying resistance to the peanut root-knot nematode.

Key words: *Arachis hypogaea* L., genetics, host resistance, *Meloidogyne* spp., molecular markers, peanut root-knot nematode.

INTRODUCTION

The worlds leading peanut producing countries include India, China and the United States. In 2013, approximately 432,000 hectares of peanuts were harvested in the United States (NASS, 2014). Root-knot

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

Abbreviation: RFLP, Restriction fragment length polymorphism.

nematodes (*Meloidogyne* spp) are the most important peanut nematode pathogens in the US inducing annual yield losses that can exceed 30% (Burrow et al., 2014); thus the monetary loss may reach well over $1 billion (Sasser and Freckman, 1987; Dickson, 1998). The three main species of *Meloidogyne* that cause damage to crop plants are: *Meloidogyne arenaria* (Neal) (Chitwood), *Meloidogyne hapla* (Chitwood), and *Meloidogyne javanica* (Chitwood) (Moens et al., 2009). The predominant pathogenic species in Southern US is *M. arenaria*, and two host races of this pathogen have been identified based on their ability (Race 1) or inability (Race 2) to reproduce on the peanut cultivar Florunner (Sasser, 1954). In the states of Alabama, Georgia, Florida and Texas, as many as 40% of the peanut fields are estimated to be infected with this pathogen (Sturgeon, 1986; Dickson, 1998). The development and deployment of root-knot nematode-resistant peanut cultivars in combination with crop rotation is currently the most effective control method used to reduce root-knot nematode damage (Collange et al., 2011). Resistance to root-knot nematodes from wild *Arachis* species has been introgressed into *Arachis hypogaea* (Choi et al., 1999; Guimarães et al., 2010).

COAN was the first peanut cultivar with a high level of resistance to root-knot nematodes and the resistance in this cultivar was derived from *Arachis cardenasii*, from a backcross introgression pathway involving an interspecific hybrid (TxAG-6) (Simpson and Starr, 1999; Church et al., 2000; Simpson and Starr, 2001). The advent of molecular markers has facilitated monitoring genes that are difficult or time-consuming to select by conventional breeding methods (Botstein et al., 1980; Lander and Botstein, 1989; Holbrook et al., 2013). The resistance in COAN is inherited as a single dominant gene and RFLP markers tightly linked to resistance locus which are easy to score and have been used in breeding programs to identify individuals homozygous for nematode resistance (Choi et al., 1999; Church et al., 2000; Chu et al., 2007; Cason et al., 2010).

There are reports identifying molecular markers linked to genes for resistance to nematodes, but limited data are available to compare the efficiency of marker-assisted selection procedures to other selection techniques (Burow et al., 1996; Burrow et al., 2014). The objective of this study was to evaluate the efficiency of marker-assisted selection using the previously identified RFLP marker for identification of individuals putatively homozygous for resistance.

MATERIALS AND METHODS

Seven University of Florida breeding populations/lines (F1334; F94x30-8-2-2-b3; F94x30-5-2-2-3-b3; F94x30-5-2-3-3-b3; F94x30-8-3-1-b2; F79x4-6; F94x30-8-2-1-b3), and the Texas A&M University breeding line TP301-1-8, were tested in field plots in 2001, at the Plant Science Unit Teaching and Research Center, University of Florida, Gainesville, FL, based on *M. arenaria* (Race 1) reproduction established protocols (Starr et al., 1995). F94x30 lines had the interspecific hybrid TxAG-6 as the parent carrying nematode resistance derived from *A. cardenasii*. Florunner and COAN were included in this test as susceptible and resistant controls, respectively. The area was previously infected and the field plots were two 5.0 m long rows with 90 cm inter rows, with four replications. Ten plants per plot were labeled from each breeding line in each from the 4 replication, total 40 plants per breeding line. After 21 days from emergency, young leaves were collected for DNA extraction from the field plot from those labeled plants. The collected plants were place in plastic bags and then transferred to 10 ml glass tubes, frozen in liquid N_2 and stored at -80°C. To extract the genomic DNA, the frozen samples were ground and DNA was extracted according to the procedure of Rogers and Bendich (1985), with modifications as follows. DNA was extracted from 1.0 g of leaves in 2X CTAB and 10X CTAB extraction buffer at 60°C followed by two chloroform/isoamyl alcohol extractions and precipitation with ice cold isopropanol. DNA precipitates were re-suspended in 100 µl high salt TE buffer and incubated at 65°C for 30 min. The supernatant was then precipitated with two volumes of ice-cold isopropanol and the pellet was washed with 80% ethanol. After drying, the DNA was re-suspended in water followed by RNAse treatment. The DNA pellet was re-suspended in TE buffer and stored at -20°C. Each sample yielded 30 to 50 µg of DNA.

Peanut DNA was quantified by spectrophotometric analysis and 20 µg was digested with *Eco*RI according to the manufacturer's instruction (New England Biolabs, Beverly, MA, USA). Digested DNA was submitted to electrophoresis (34 V) for 16 h on 0.8% agarose gels and transferred to Hybond N+ membrane (Amersham, Arlington Heigts, IL) by capillary blotting (Southern, 1975; Chittenden et al., 1994) and UV cross linked or 3 min at 1600 nm. Dr. Gregory T. Church, from Texas A & M University, provided the R2430E probe, which is 4.2 cM distant from the resistance locus (Choi et al., 1999; Church et al., 2000; Church et al., 2001), which was prepared by PCR amplification. Fifty nanograms of probe DNA was labeled with α-P^{32} dCTP by random primer extension (Feinberg and Vogeltein, 1983). Pre-hybridization and hybridization were performed at 65°C with 7% SDS and denatured salmon sperm DNA (Church and Gilbert, 1984). Samples were washed three times for 20 min each at 65°C with 0.5X SSC and 0.1% SDS. Hybridized blots were auto radiographed using X-ray film (Kodak XAR-5) and two intensifying screens at -70°C for 10 days.

Individuals were scored as homozygous (RR) for resistance if only the band associated with resistance was present; heterozygous (Rr) for resistance if the band associated were present; and susceptible (rr) if the band associated with resistance was absent. The resistance to *M. arenaria* race 1 was measured based on egg masses and galls present on peanut roots and pegs. Nematode reproduction was measured in the same 10 plants previously labeled in each replication and used for the RFLP screening. Two weeks prior to crop maturity, plants were harvested and the soil was washed from the roots with tap water. Roots were then placed into 300 ml beakers containing approximately 900 ml of 0.05% Phloxine B solution for 3 to 5 min (Daykin and Hussey, 1985). To each plant, a root-gall and an egg-mass rating was assigned. Each plant was rated according to the number of egg masses and galls found on roots, pegs and pods. A plant given a rating of 1 (no galls or egg masses on roots, pegs and pods) was considered highly resistant, a plant rated as 2 (1 to 10 egg masses and/or galls on roots and less than 10 egg masses and/or galls on pegs and pods) was considered resistant, a rating of 3 (11 to 100 egg masses and/or galls on roots and between 10 to 50 egg masses and/or galls on pegs and pods) indicated that the plants were susceptible and a plant rated as 4 (> 100 egg masses and/or

Table 1. *Meloidogyne arenaria* reproduction at field assay in peanut breeding population which has the interespecific hybrid TxAG-6 as parent for nematode resistance, the resistant breeding line TP301-1-8, the resistant COAN genotype; and the susceptible Florunner genotype.

Peanut genotypes	Galls and eggs mass index (average ± standard error)	% egg mass on pods and pegs	Classification[1]
F1334	3.40 ± 0.52bc[2]	11-50	S
F94x30-8-2-2-b3	3.50 ± 0.71abc	11-50	S
F94x30-5-2-2-3-b3	3.80 ± 0.42ab	11-50	S
F94x30-5-2-3-3-b3	3.70 ± 0.48 ab	11-50	S
F94x30-8-3-1-b2	3.10 ± 0.32 c	11-50	S
F94x4-6	3.80 ± 0.42 ab	11-50	S
F94x30-8-2-1-b3	3.90 ± 0.31 ab	11-50	S
Florunner	4.00 ± 0.00a	>50	HS
TP301-1-8	1.00 ±0.00 d	0	R
COAN	1.00 ± 0.00 d	0	R

CV=13.51%. [1] HS= High susceptible, R= resistant, S=susceptible. [2] Means within a column followed by the same letter are not statistically different at P=0.05 based on Tukey's multiple range test.

galls on roots and > 50 egg masses and/or galls on pegs and pods) was considered highly susceptible. Nematode count data were subjected to analysis of variance and Tukey test using the SAS statistics program (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

In the field tests to confirm resistant genotypes based on RFLP analysis, no evidence of segregation was found in the breeding materials for the *M. arenaria* resistance locus. The gall and egg mass index for the resistant cultivar COAN was 1, whereas the mean gall and egg mass index on roots and pods of the susceptible cultivar Florunner was 4.00 (Table 1). Reproduction of *M. arenaria* on each of the lines was measured by the number of galls and egg masses on roots, ranged from 0 to greater than 100 and, as measured in percentages, ranged from 0 to > 50% galls and egg masses on pods and pegs. The breeding populations had a gall and egg mass index ranging from 1.00 to 3.90 with greater than 11% egg masses on pods and pegs, which was more than the reproduction found on Florunner ($P<0.001$) (Table 1). TP301-1-8 had no nematode parasitism with a gall and egg mass index equal to 1.0 on roots and pods, which was not significantly different ($P>0.05$) from the reproduction on COAN (Table 1). TP301-1-8 has a single gene for resistance with an *RR* genotype (Simpson and Starr, 2001). In previous report, Church et al. (2000) identified 29% of the TP301-1-8 breeding line as homozygous for resistance, using the RFLP probe R2430E. In a study to identify RFLP markers linked to resistance to *M. arenaria* in six BC_5F_2 peanut breeding populations derived from the interespecific hybrid TxAG-7, Choi et al. (1999) estimated that resistance was conditioned by a single dominant gene.

The RFLP probe R2430E used for screening was linked to high levels of nematode resistance. The resistant and susceptible alleles were quite distinct and easy to score (Figure 1). All populations with a susceptible RFLP genotype (Figure 1) had a susceptible phenotype based on nematode reproduction (Table 1). Previously, R2430E was shown to be 4.2 centiMorgans (cM) from the nematode resistance locus (Church et al., 2001).

In the present investigation, the RFLP probe R2430E was effective in identifying homozygous individuals resistant to *M. arenaria* Race 1, with a high level of confidence. An advantage of using this marker is the opportunity to screen peanut genotypes without relying on inoculation tests with the pathogen, which is cumbersome and time-consuming. Marker-assisted selection also allowed an evaluation of resistance to be performed three months prior to the measurement of nematode reproduction. In addition to saving time, the use of marker-assisted selection for nematode resistance will reduce the cost associated with planting and maintenance, by reducing the number of field plantings to those plants actually carrying the resistance gene in subsequent field trials.

Conflict of Interests

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

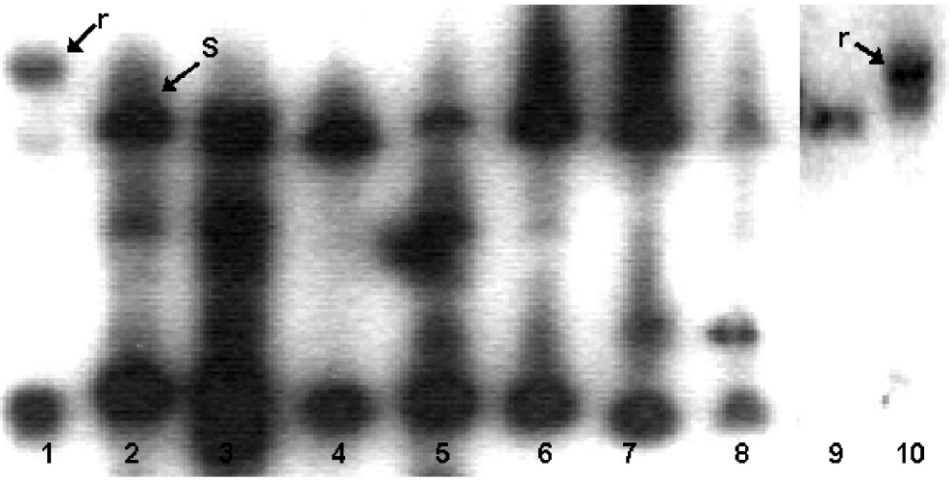


Figure 1. RFLP locus R2430E linked to resistance to *Meloidogyne arenaria* in peanut breeding lines. r = resistant and s = susceptible alleles. Lane 1 is the control resistant COAN genotype; lane 2 is the control susceptible Florunner genotype; lanes 3 to 9 are the breeding lines F1334; F94x4-6; F9430-8-2-1-b3; F94x30-8-3-1-b2; F94x30-8-2-2-b3; F94x30-5-2-2-3-b3, F94x30-5-2-3-3-b3, respectively; line 10 is the resistant breeding line TP301-1-8.

REFERENCES

Botstein D, White RL, Skolnic M, Davis RW (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. 32:314-331.

Burow MD, Simpson CE, Paterson AH, Starr JL (1996). Identification of peanut (*Arachis hypogaea* L.) RAPD markers diagnostic of root-knot nemattode (*Meloidogyne arenaria* (Neal) Chitwood) resistance. Mol. Breed. 2:369-379.

Burrow MD, Starr JL, Park CH, Simpson CE, Paterson AH (2014). Introgression of homeologous quantitative trait loci (QTLs) for resistance to the root-knot nematode [*Meloidogyne arenaria* (Neal) Chitwood] in an advanced backcross-QTL population of peanut (*Arachis hypogaea* L.). Mol. Breed. 1-14.

Cason JM, Simpson CE, Starr JL, Burow MD (2010). Marker-assisted selection in the transfer of root-knot nematode resistance in the commercial peanut (*Arachis hypogaea* L.). Tex. J. Sci. 62:49-58.

Chittenden LN, Schertz KF, Lin YR, Wing RA, Paterson AH (1994). A detail RFLP map of *Sorghum bicolor* x *S. propinquum*, suitable for high-density mapping suggests ancestral duplication of Sorghum chromosomes or chromosomal segments. Theor. Appl. Genet. 87:925-933.

Choi KM, Burow D, Church GB, Paterson AH, Simpson CE, Starr JL (1999). Genetics and mechanism of resistance to *Meloidogyne arenaria* in peanut germplam. J. Nematol. 31:283-290.

Church GM, Gilbert W (1984). Genomic sequencing. Proc. Natl Acad. Sci. USA. 88:1991-1995.

Church GT, Simpson CE, Burow MD, Paterson A H, Starr JL (2000). Use of RFLP marker for identification of individuals homozygous for resistance to *Meloidogyne arenaria* in peanut. Nematology 5:575-580.

Church GT, Simpson CE, Burow MD, Starr JL (2001). Identification of RFLP loci flanking a nematode resistance locus in peanut. Phytopathology 91:S17.

Chu Y, Holbrook CC, Timper P, Ozias-Akins P (2007). Development of a PCR-Based Molecular Marker to Select for Nematode Resistance in Peanut. Crop Sci. 47(2): 841-845.

Collange B, Navarrete M, Peyre G, Mateille T, Tchamitchian M (2011). Root-knot nematode (*Meloidogyne*) management in vegetable crop production: The challenge of an agronomic system analysis. Crop Prot. 30 (10):1251-1262.

Daykin ME, Hussey RS (1985). Staining and histopathological techniques in nematology. In: Barker KR, Carter CC, Sasser JN (Eds.) An advanced treatise on Meloidogyne. Vol. II: Methodology. Raleigh, North Carolina State University. pp. 39-48.

Dickson DW (1998). Peanut. In: Barker KR, Peterson GA, Windham GL (Eds.). Plant and nematode interaction. Madison, WI: American Society of Agronomy. pp. 523-566.

Feinberg AP, Vogelstein B (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.

Guimarães PM, Brasileiro ACM, Proite K, Araujo ACG, LealBertioli SCM, Pic-Taylor A, Silva FR, Morgante CV, Ribeiro SG, Bertioli DJ (2010). A study of gene expression in the nematode resistant wild peanut relative, *Arachis stenosperma*, in response to challenge with *Meloidogyne arenaria*. Trop. Plant Biol. 3:183-192.

Holbrook CC, Isleib TG, Ozias-Akins P, Chu Y, Knapp SJ, Tillman B, Guo B, Gill R, Burow MD (2013). Development and Phenotyping of Recombinant Inbred Line (RIL) Populations for Peanut (*Arachis hypogaea*). Peanut Sci. 40(2):89-94.

Lander ES, Botstein D (1989). Mapping mendelian factors under-lying quantitative traits using RFLP linkage maps. Genetics 121:185-199.

Moens M, Perry RN, Starr JL (2009). Meloidogyne species a diverse group of novel and important plant parasites. In: Perry RN, Moens M, Starr JL (Eds.) Root-Knot Nematodes. Wallingford, Oxford shire: CAB International. pp. 1-17.

National Agricultural Statistics Service - NASS (2014). http://www.nass.usda.gov.

Rogers W, Bendich AJ (1985). Extration of DNA from milligram amounts of fresh, herbarium and mummified tissues. Plant Mol. Biol. 5:69-76.

Sasser JN (1954). Identification and host-parasite relationships of certain root-knot nematodes (*Meloidogyne* spp.) Maryland Agricultural Experiment Station Bulletin, A-77:1-13.

Sasser JN, Freckman DW (1987). A world perspective of nematology: the role of society. In: Veech JA, Dickson DW (Eds.) Vistas on nematology. Hyatsville, MD: Society of Nematologists. pp. 7-14.

Simpson CE, Starr JL (1999). Development and realease of root-knot nematode resistant peanut variety. Proceedings of the American Peanut Research and Education Society 31:68.

Simpson CE, Starr JL (2001). Registration of 'COAN Peanut'. Crop Sci. 41:918.
Southern EM (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.
Starr JL, Simpson CE, Lee Jr TA (1995). Resistance to *Meloidogyne arenaria* in advanced generation breeding lines of peanut. Peanut 22:59-61.
Sturgeon Jr RV (1986). Peanut disease loss estimates for major peanut producing states in the United States for 1984 and 1985. Proceedings of the American Peanut Research and Education Society 18:24-26.

academicJournals

Vol. 13(26), pp. 2613-2625, 25 June, 2014
DOI: 10.5897/AJB2014.13661
Article Number: 4461A2545606
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic divergence of roundup ready (RR) soybean cultivars estimated by phenotypic characteristics and molecular markers

Otávia Tiago Villela, Sandra Helena Unêda-Trevisoli*, Fabiana Mota Da Silva, Laerte Souza Bárbaro Junior and Antonio Orlando Di Mauro

Department of Plant Production, FCAV/UNESP-Jaboticabal- São Paulo State, Brazil.

Received 27 January, 2014; Accepted 16 June, 2014

The aim of this study was to estimate the genetic diversity in 74 RR soybean cultivars from different Brazilian breeding programs. Analyzes were based on multivariate statistical techniques from phenotypic characteristics and microsatellite molecular markers (SSR). Ten agronomic traits were used in the analysis of the Euclidean distance, Tocher's clustering, UPGMA clustering and principal component analysis. Eighty-six of 100 SSR primer-pairs studied were selected based on their polymorphism information content, and analyzed using Jaccard Coefficient and UPGMA clustering method. The cultivars were clustered into seven groups according to the UPGMA and Tocher's methods, based on agronomic traits, while molecular analysis identified six groups. The phenotypic distances ranged from 0.46 to 9.79 and the dissimilarity measurements, based on SSR molecular markers, ranged from 0.07 to 0.73. Both results from agronomic traits and molecular markers showed that there is genetic variability among the RR cultivars and that the Monsanto breeding program has the most divergent germplasm. The analyzed agronomic traits and the chosen SSR markers were effective in assessing the genetic diversity among genotypes, besides proving to be useful for characterizing genetic variability of soybean germplasm.

Key words: *Glycine max*, genetic variability, phenotypic characteristic, SSR markers.

INTRODUCTION

Soybean (*Glycine max* (L.) Merrill) is one of the most important commodities grown and commercialized in the world, and Brazil is currently the second largest producer with 90% of its area (24.3 million hectares) planted with GMO soybean cultivars (James, 2013). With the introduction of GMO soybean resistant to Roundup

*Corresponding author. E-mail: strevisoli@fcav.unesp.br. Tel: (55) 16- 3209-2666.

Abbreviations: CTAB, Cetyl trimethylammonium bromide; **GMO,** genetically modified organism; **PCR,** polymerase chain reaction; **SSR,** simple sequence repeat; **UPGMA,** unweighted pair group method with arithmetic mean; **RR soybeans,** roundup ready soybeans.

Ready herbicide, various public and private seed breeding companies have incorporated the RR gene into their best lines (Green, 2009). Despite the high number, the Brazilian soybean cultivars are extremely uniforms because they originated from only a few ancestral lines, which resulted in a narrow genetic base of germplasm (Miranda et al., 2007; Priolli et al., 2010; Wysmierski and Vello, 2013). This fact, together with the lack of genetic variability, brings risks for cultivars productivity levels and susceptibility to pests, pathogens and environmental stresses. The use of single resistance genes in a monoculture is a source of strong selective pressure for pathogen races capable of overcoming the resistance and also can influence the maintenance of cultivars to cope with multiple environmental stresses and changing conditions (Hajjar et al., 2008). Considering the importance of GMO soybeans for Brazilian production and the need to develop new more productive genotypes adapted to different environments, the study of the genetic diversity of RR cultivars is very important for knowing the existing variability among them and also within the breeding programs which produced them. Estimates of genetic divergence through multivariate analysis of both agronomic traits and molecular markers can supply information on the genetic variability of germplasm of various crops (Jose et al., 2009; Schuster et al., 2009; Liu et al., 2011). Multivariate techniques, such as discriminatory analysis, principal components, coordinate and cluster analysis, may be used to study genetic diversity and they have been very useful for unifying information from a series of variables related to genetic breeding.

The study of genetic diversity based on agronomic traits, mainly the quantitative ones, is indispensable considering their economic importance and the need to select superior parents. Multivariate analysis based on phenotypic data has been used to access genetic diversity of soybean (Mikel et al., 2010; Salimi et al., 2012; Peluzio et al., 2012); has also occurred with many of plants species, such as bean (Chiorato et al., 2007), cotton (Li et al., 2008) and rubber tree (Gouvêa et al., 2010).

More recently, with molecular marker technology, it has been possible to access species genotype and detect genetic variations at the DNA, which are inherited genetically. The microsatellite markers or Simple Sequence Repeats (SSR) are widely distributed throughout genomes and, can be highly polymorphic, for this reason have been successfully used to infer about genetics, phylogeny, pedigree, and identity of traits and germplasm accessions. The high level of polymorphism detected increases the resolution of the study of genealogy and genetic diversity and reduces the number of markers required to distinguish genotypes. SSR markers have been used to analyze genetic diversity in several species, including maize (Laborda et al., 2005), wheat (Huang et al., 2007) and soybean (Yamanaka et al., 2007; Mulato et al., 2010; Singh et al., 2010).

The objective of this study was to evaluate the genetic divergence among RR soybean cultivars from different breeding programs, using phenotypic data and SSR molecular markers.

MATERIALS AND METHODS

A group of 74 OGM soybean cultivars were selected to represent distinct geographical regions of Brazil. The cultivars chosen belong to public and private soybean breeding companies, which develop and commercialize the Roundup Ready technology in the country. The cultivars were numbered from 1 to 74 (Table 1), corresponding to the identification of the genotype throughout the work.

Analysis of phenotypic data

The field experiment was set up in the crop year 2011/2012 at the Fazenda de Ensino, Pesquisa e Extensão da Faculdade de Ciências Agrárias e Veterinárias (FCAV-UNESP), in Jaboticabal, São Paulo State, Brazil. The experimental area was homogeneous and each block consisted of a single 5 m row, with 0.5 m spacing between rows. This design was adopted owing to the small number of available seeds and according to the methodology used by germplasm banks, where the genotypes are planted in single rows, without repetitions (Carvalho et al., 2003; Chiorato et al., 2007). The agronomic traits were evaluated using the mean data of six plants collected at random within the block. Average values of ten agronomic traits, each based on six replicates, were subjected to multivariate analysis. They comprised: 1) number of days to flowering, 2) number of days to maturity, 3) first pod insertion height, 4) plant height at maturity, 5) lodging, 6) agronomic value, 7) number of branches, 8) number of pods, 9) weight of 100 seeds and, 10) grain productivity. The Euclidean distance was used to calculate the genetic distance among cultivars. The dissimilarity matrix was analyzed using Tocher's clustering and the method of average linkage between groups, UPGMA, in an attempt to establish cultivar groups. A principal component analysis was later used to evaluate the contribution of each variable to genetic divergence. Statistical analyses were performed using the Genes software (Cruz, 2008) and UPGMA dendrogram was constructed using Statistica software (Statsoft, 2004).

Analysis of molecular data

The genomic DNA samples were extracted from young trifoliate leaf tissues using the CTAB method, as described by Ferreira and Grattapaglia (1998). One hundred SSR primer-pairs distributed along all the 20 linkage groups of soybean were selected based on the information contained in the soybean genetic map, to provide efficient coverage of the whole genome. PCR amplifications were carried out in a 25 μl final volume containing 12 ng of genomic DNA, 4 mM $MgCl_2$, PCR 1X buffer (50 mM HCl, 10 mM Tris-HCl, pH 8.0), 200 μM of DNTP mixture, 1 U Taq DNA polymerase and 10 μM of each forward and reverse primer. A specific annealing temperature (Ta) was calculated for each SSR. The thermocycling program was composed of an initial denaturation cycle of 7 min at 94°C, followed by 32 cycles of 1 min at 94°C, 1 min at the specific annealing temperature of each primer-pair and extension of 2 min at 72°C, followed by a final elongation step of 7 min at 72°C. Amplification fragments were separated by electrophoresis on 3% agarose gels, with a TBE 1X buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). Gels were stained with ethidium

Table 1. RR soybean cultivars used in phenotypic and molecular analyses and the respective breeding programs which developed them.

Breeding programs	Cultivar	Breeding programs	Cultivar
Agroeste	1. AS7307 RR	**FT Sementes**	35. FTS Jaciara RR
	2. AS8380 RR		
Brasmax	3. BMX Apolo RR		36. GB874 RR
	4. BMX Energia RR		37. M7211 RR
	5. BMX Força RR		38. M7578 RR
	6. BMX Impacto RR		39. M7639 RR
	7. BMX Magna RR		40. M7908 RR
	8. BMX Potência RR	**MONSANTO**	41. M8230 RR
	9. BMX Titan RR		42. M8336 RR
Coodetec	10. CD214 RR		43. M8360 RR
	11. CD219 RR		44. M8527 RR
	12. CD230 RR		45. M8766 RR
	13. CD242 RR		46. M9144 RR
	14. CD243 RR		47. MSOY7878 RR
Emgopa	15. EMGOPA315 RR	**Nidera**	48. A4910 RR
			49. A6411 RR
Embrapa	16. BRS243 RR		50. NA7255 RR
	17. BRS244 RR		51. NA8015 RR
	18. BRS246 RR	**Pioneer**	52. P98Y11 RR
	19. BRS278 RR		53. P98Y12 RR
	20. BRS279 RR		54. P98Y30 RR
	21. BRS8160 RR		55. P98Y51 RR
	22. BRS8460 RR		56. P98Y70 RR
	23. BRSMG740S RR		57. P98Y31 RR
	24. BRSMG750S RR		58. P98Y01 RR
	25. BRSMG760S RR	**Soy Tech Seeds**	59. STS810 RR
	26. BRSMG850G RR		60. STS820 RR
	27. BRSMG811C RR		
	28. BRS Baliza RR		61. NK7074 RR
	29. BRS Charrua RR		62. SYN9074 RR
	30. BRS Favorita RR		63. SYN9078 RR
	31. BRS Juliana RR		64. ANTA82 RR
	32. BRS Pampa RR		65. TMG103 RR
	33. BRS Silvânia RR		66. TMG106 RR
	34. BRS Valiosa RR	**Syngenta**	67. TMG108 RR
			68. TMG115 RR
			69. TMG123 RR
			70. TMG132 RR
			71. TMG1179 RR
			72. TMG1182 RR
			73. TMG4001 RR
			74. TMG7188 RR

bromide to visualize bands. Data on the presence (1) or absence (0) of SSR bands were transformed into genotypic data in order to identify loci and alleles. The polymorphic information content (PIC) value for each SSR locus was calculated using the following

Table 2. 15 pairs of the most divergent and similar cultivars estimated from the Euclidean distance.

Order	15 most divergent pairs	
	Euclidean distance	Pairs of cultivars
1º	9.79	31-49
2º	9.73	6-31
3º	9.71	4-31
4º	9.64	3-31
5º	9.38	8-31
6º	9.34	48-31
7º	9.14	14-40
8º	9.08	45-49
9º	8.93	9-31
10º	8.87	40-45
11º	8.80	45-48
12º	8.70	10-31
13º	8.66	4-45
14º	8.65	3-45
15º	8.64	9-45
	15 most similar pairs	
Order	**Euclidean distance**	**Pairs of cultivars**
1º	0.46	3-4
2º	0.84	1-37
3º	0.86	1-64
4º	1.13	21-30
5º	1.14	1-50
6º	1.23	11-52
7º	1.25	4-49
8º	1.27	3-49
9º	1.28	6-49
10º	1.31	36-56
11º	1.32	2-47
12º	1.37	53-30
13º	1.41	4-6
14º	1.43	54-65
15º	1.48	3-6

formula:

$$PIC = 1 - \sum_{j=1}^{n} P_{ij}^{2}$$

Where, P_{ij} is the frequency of the allele j on the marker i.

The similarity measurements based on the SSR markers were calculated from the Jaccard Coefficient and converted into dissimilarity through arithmetic complement ($d_{ii'}$), with: $d_{ii'} = 1 - S_{ii'}$. A genetic distance matrix was estimated using Genes software (Cruz, 2008). Cluster analyses were performed using UPGMA method with the Statistica software (Statsoft, 2004). Clustering stability was tested by the Bootstrap procedure based on 10.000 re-sampling using the BooD program (Coelho, 2002). The dissimilarity matrices from the phenotypic and molecular data were correlated using the Genes software (Cruz, 2008). Both t and Mantel tests were employed with 10.000 simulations to attribute significance values to the data.

RESULTS AND DISCUSSION

Phenotypic diversity

The genetic distance among cultivars obtained through agronomic traits ranged from 0.46 to 9.79, indicating the presence of genetic variability among soybean cultivars (Table 2). Several authors have also found genetic variability between RR Brazilian soybean cultivars for many agronomic traits (Viera et al., 2009; Santos et al., 2011; Peluzio et al., 2009). In Table 2, there are the 15 pairs of the most divergent and similar cultivars identified on the dissimilarity matrix. The maximum Euclidean distance (dii' = 9.79) was observed between the BRS Juliana and A6411, also, among the most divergent combinations found, BRS Juliana was present in the major part. The minimum distance (dii' = 0.46) was found between cultivars BMX Apolo and BMX Energia, both belonging to the same breeding program (Brasmax). Diversity within breeding programs was evaluated for those programs that had more than five cultivars (Brasmax, Coodetec, Embrapa, Monsanto, Pioneer and TMG). The maximum and minimum distances between cultivars within their respective breeding programs were identified (Table 3). The Brasmax breeding program showed the lowest distance between cultivars (0.46). It also presented the lowest distance (3.35) between cultivars when evaluating the maximum distances among programs. The genetic similarity among Brasmax cultivar may be due to the extensive use of their best lines as parents for transferring and incorporating the RR gene. The wide variation among distance measurements indicates dissimilarity between cultivars, as well as variability among them. These results agree to that verified by Liu et al. (2011) and Malik et al. (2007), when assessed diversity among soybean cultivars is using phenotypic characteristics.

According to Sneller (2003), the advent of RR cultivars has had little impact on diversity, once this technology was widely used by many programs. However, to Mikel et al. (2010), facilitating gene transferences by replacing the two-parent breeding cross by partial backcrosses the genetic diversity within breeding programs was probably compromised. The Tocher's cluster analysis, based on genetic dissimilarity measurements, separated the 74 soybean cultivars into seven groups, where three of these consisted of a single cultivar (Table 4).

The Group I contained most of the cultivars evaluated (74.3% of the total), including at least one cultivar from each breeding program. This fact shows similarity among soybean cultivars, even coming from different breeding

Table 3. Minimum and maximum distances observed between cultivars belonging to the same genetic breeding programs.

Breeding programs	Mínimum		Maximum	
	Euclidean distance	Pairs	Euclidean distance	Pairs
Brasmax	0.46	3-4	3.35	6-9
Coodetec	2.51	10-12	6.04	10-14
Embrapa	1.13	21-30	8.43	29-31
Monsanto	1.71	37-47	8.87	40-45
Pioneer	1.55	55-56	6.79	53-58
TMG	1.93	65-72	6.68	67-68

Table 4. Clustering of the 74 RR soybean cultivars according to agronomic data, using Tocher's method based on Euclidean distance.

Group	RR soybean cultivars
I	3, 4, 49, 6, 8, 7, 48, 10, 5, 9, 12, 29, 18, 64, 1, 47, 24, 50, 37, 16, 39, 11, 52, 2, 38, 13, 71, 21, 53, 30, 61, 42, 22, 55, 23, 15, 57, 63, 54, 56, 74, 43, 27, 62, 73, 17, 36, 72, 66, 69, 35, 25, 65, 51
II	34, 68, 40, 44, 70, 46, 33
III	32, 41, 28, 60, 59, 67
IV	19, 45, 58
V	20
VI	31
VII	14

programs. This similarity is probably due to the RR gene introduction into the cultivars by the companies. The RR gene was engineered into the soybean cultivar to produce line 40-3-2, which is highly tolerant of glyphosate (Sneller, 2003). This line was used as a donor parent in traditional breeding schemes to develop RR soybean cultivars, which is used by many programs and could explains the clustering of cultivars.

Group II contained 9.4% of the cultivars, Group III 8.1% and Group IV 4.0%. The BRS279, BRS Juliana and CD243 cultivars were isolated into Groups V, VI and VII respectively, indicating that these cultivars are the most divergent. The formation of these groups is of major importance for choosing parents in breeding programs, once the cultivars in more distant groups are dissimilar and may be considered promising to develop new cultivars (Peluzio et al., 2009). Considering each breeding program, the Embrapa cultivars were distributed into six groups (I, II, III, IV, V and VI), the Monsanto cultivars into four (I, II, III and IV) and the TMG cultivars into three (I, II and III). The formation of various groups indicates the existence of genetic diversity between cultivars within the breeding programs since one of the characteristics of Tocher's classification is homogeneity within and heterogeneity between groups (Cruz, 2008). Several authors have shown similar results. Within this context, Peluzio et al. (2012) and Shadakshari et al. (2011) have used the Tocher's optimization method to estimate the diversity among genotypes evaluated by agronomic traits. Reina et al. (2014), also based on agronomic traits and using Tocher method, verified 11 cultivars separated into four genetically distinct clusters.

The genetic diversity among the seven Pioneer cultivars and among the five Coodetec cultivars was smaller, with most of the cultivars, except for P99R01 and CD243, allocated in the Group I. All the Brasmax cultivars were allocated in the Group I. The lower number of cultivars evaluated from the Pioneer, Coodetec and Brasmax programs may have resulted in the smaller genetic diversity observed. However, this factor was not important when only these three programs were compared since despite the higher number of Brasmax cultivars, they were all allocated to a single group. The UPGMA clustering, which is represented by a dendrogram (Figure 1), also resulted in the formation of seven distinct groups. Group I contained 52 cultivars, which represented 72.2% of the total cultivars evaluated. The group II was formed by 3 cultivars (4%), group III contained only 2 cultivars (2.7%). Groups IV and VI were formed by 10 and 5 cultivars, representing 13.5 and 6.75%, respectively. The cultivars CD243 and BRS Juliana formed the isolated groups V and VII, respectively.

Miranda et al. (2007) studying the genetic structure of 90 elite soybean cultivars adapted to different Brazilian environments, have concluded that the UPGMA method

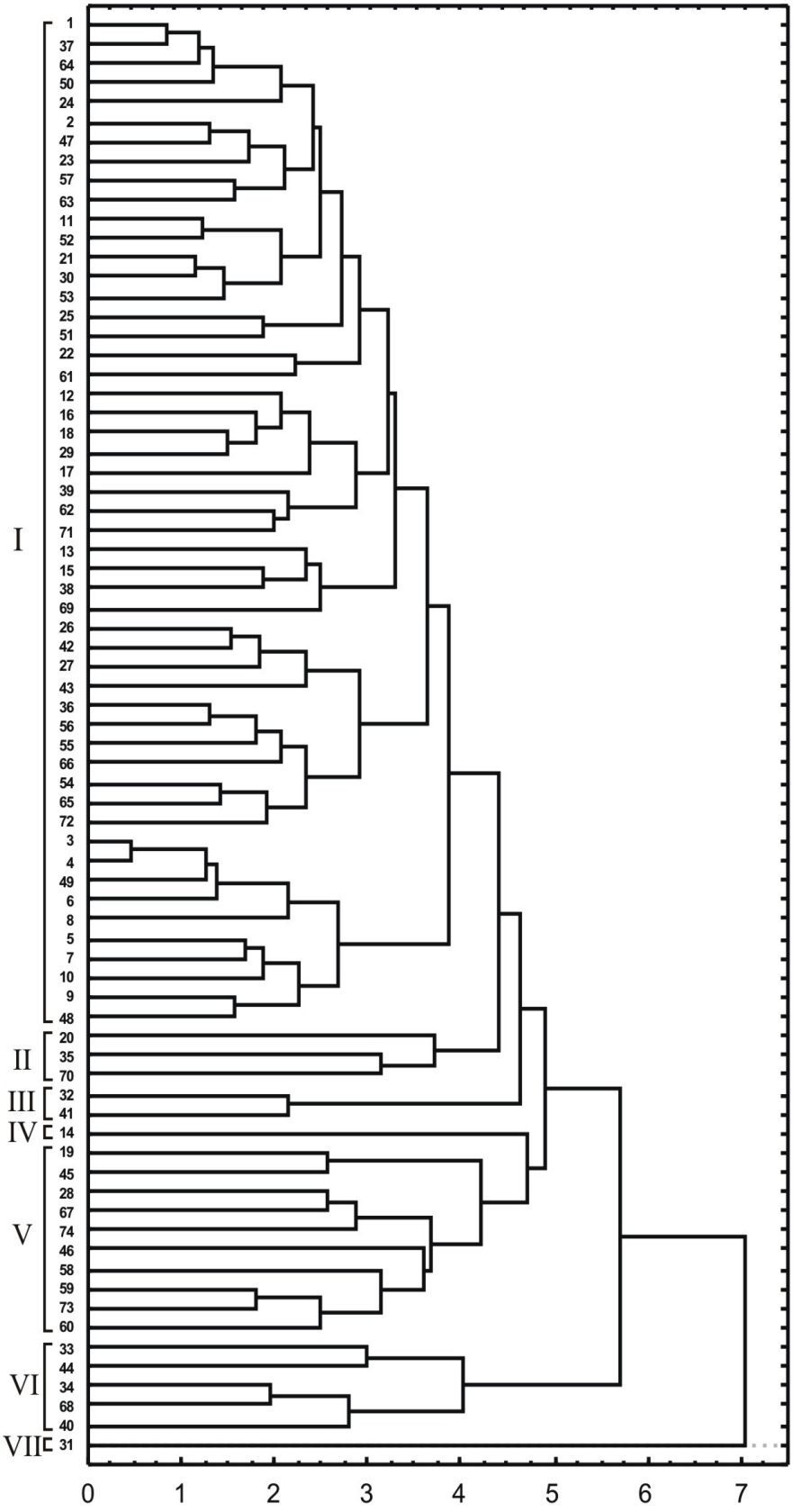


Figure 1. Dendrogram obtained by the UPGMA method, representing the genetic dissimilarity between 74 RR soybean cultivars, based on 10 agronomic traits.

was efficient for clustering the cultivars in several groups, according to their common ancestral. Moreover, this method was also efficient to demonstrate the genetic structure of the main Brazilian cultivars. The Tocher's method and UPGMA hierarchical method agreed among themselves on groups' constitution. Predominantly, the classification of genotypes between the two methods has coincided, with some exceptions such as FTS Jaciara, TMG132, TMG4001 and TMG7188 belonging to different groups and BRS 279 which was not isolated by the dendrogram analysis. In relation to diversity within the breeding programs, the only difference on groups formation was found among TMG program cultivars, which were divided into four groups (I, II, IV and VI) and not three, as was observed in the Tocher's analysis. Similarity between the clusters obtained by the Tocher's method and UPGMA hierarchical methods have also been observed by several authors studying genetic diversity in different crops, resulting in good information on the genotypes evaluated (Arshad et al., 2006; Beyene et al., 2005; Liu et al., 2011; Salimi, 2013).

Santos et al. (2011) concluded that the UPGMA and Tocher's cluster methods also agreed among them for 48 genotypes clustered into four groups. The dendrogram demonstrated the distances among genotypes, and as a result, it is possible to identify BMX Apolo and BMX Energia (both belonging to Brasmax program) as the most similar cultivars. As verified by the isolation of group VII, BRS Juliana cultivar was the most divergent and with the largest distance in the last level, when compared to 73 others. By using the dendrogram, it is possible to evaluate the groups formation and, consequently, to select genetically distinct cultivars. Studying the phenotypic diversity, Cui et al. (2001) distinguished Chinese and Americans soybean cultivars using the UPGMA methodology. Also, with the same methodology Liu et al. (2011) clustered in 5 groups, 91 cultivars belonging to Shaanxi province. By the principal components analysis it is possible to assess the genetic diversity and the influence of each characteristic for the differentiation of genotypes. The analysis of the ten agronomic traits showed that four components absorbed 80.84% of the total accumulated variation. The results of present studies are agree with those of Narjesi et al. (2007), which reported that five principal components for 30 soybean genotypes explained 80.2% variation of all data. The first principal component accounted for 36.65% of the observed variation, and the trait with the largest contribution to the diversity of cultivars was number of days to flowering. The second principal component explained 21.19% of variation and the mainly contributor was grain productivity. The third and fourth principal component absorbed 15.36 and 7.63% of the variation that were due to agronomic value and number of days to maturity, respectively.

Our result corresponded well with the study of Salimi (2013), who analyzed genetic diversity 19 soybean genotypes using agronomic traits and also showed that the number of day to flowering was the major contributor to difference cultivars. Moreover, Peluzio et al. (2009) and Shadakshari et al. (2011) also observed that number of days to flowering, grain yield and number of days to maturity were those that most contributed to differentiate genotypes.

Diversity based on molecular markers

Eighty-six of the 100 SSR markers analyzed were polymorphic and informative to evaluate the 74 cultivars (Table 5). A total of 195 alleles were identified using the polymorphic SSR primer-pairs. The number of alleles per locus ranged from 2 to 4, with a mean of 2.3. Similar results were showed by Bizari et al. (2014) when 46 soybean genotypes were evaluated, with 75 SSR primers, and found 173 alleles with a mean of 2.3 alleles per locus. Li et al. (2008), found a total of 121 alleles, generated by 35 SSR primers across 101 genotypes, and the range of allele per SSR primer was from 1 to 7 with an average of 3.45. Polymorphic information content, a reflection of allelic diversity and frequency among the soybean cultivars analyzed were generally high for all the SSR loci tested (Table 5). PIC values ranged from 0.04 (Satt 277) to 0.72 (Satt 308), with an average of 0.42. These results indicate that the selected microsatellites are very informative among the cultivars. The polymorphism of SSR loci detected in this study were in agreement with the data of Singh et al. (2010) and Tantasawat et al. (2011), who detected mean gene diversity values of 0.50 and 0.60 in a group of 44 and 25 soybean genotypes, respectively. However, these results were lower than that reported by Wang et al. (2006), who obtained PIC values ranging from 0.5 to 0.92 with a mean of 0.78, when analyzing 129 accessions of soybeans. Various authors have described the efficiency of SSR markers when analyzing genetic diversity (Fu et al., 2007; Kuroda et al., 2009; Guan et al., 2010), also were observed in the present work, whose the SSR markers selected were informative and useful for studies of genetic diversity in soybeans. Studying genetic variability in 105 soybean accessions, Shi et al. (2010) used 65 SSR primer-pairs and Mulato et al. (2010), evaluating 79 soybean accessions from different regions of the world, found a high genetic diversity among them using only 30 SSR primers.

The pairwise genetic dissimilarity between cultivars, calculated using Jaccard's similarity coefficient, varied from 0.07 to 0.73. The lowest distance was observed between BMX Força and BMX Potência (0.07) while the greatest distance occurred between BMX Titan and M7578 (0.73). The maximum and minimum dissimilarity measures found within the breeding programs of Brasmax, Coodetec, Embrapa Monsanto, Pioneer and TMG were listed in Table 6. The Brasmax breeding

Table 5. Number of polymorphic SSR primers used to evaluate 74 RR soybean cultivars, linkage group (LG), motif of repetition, chromosome number, specific temperature of each primer-pair (Ta), and number of alleles observed and values of polymorphic information content (PIC).

Number	SSR	LG	Cr	Motif	Ta (°C)	Alleles	PIC
1	SAT_001	D2	17	(AT)38	59	2	0.50
2	SAT_097	A2	8	(AT)30	52	3	0.60
3	SAT_141	G	18	(AT)11C(GA)12	49.5	2	0.44
4	SAT_250	A2	8	(AT)19	62	2	0.46
5	SATT 014	D2	17	(TTA)8	53	2	0.08
6	SATT 020	B2	14	(AAT)16	47	3	0.41
7	SATT 022	N	3	(TAT)17	58	3	0.60
8	SATT 041	D1b	2	(AAT)17	47	2	0.28
9	SATT 045	E	15	(AAT)18	44	2	0.38
10	SATT 066	B2	14	(ATT)28	48	2	0.50
11	SATT 070	B2	14	(ATT)24	47	3	0.66
12	SATT 080	N	3	(ATT)23	45.7	2	0.46
13	SATT 094	O	10	(TAT)15TG(TTA)4	47	2	0.44
14	SATT 100	C2	6	(TTA)13	47	2	0.18
15	SATT 129	D1a	1	(AAT)25	57	3	0.63
16	SATT 141	D1b	2	(ATA)25	58	3	0.59
17	SATT 154	D2	17	(TAT)7CATC(ATT)20A(CTG)4	50	2	0.49
18	SATT 166	L	19	(TTA)19	52	2	0.42
19	SATT 173	O	10	(TAT)18	52	3	0.60
20	SATT 174	A1	5	(TTA)10	55	2	0.37
21	SATT 180	C1	4	(TAT)16	42	2	0.37
22	SATT 184	D1a	1	(ATT)14(TTG)5	45	3	0.51
23	SATT 185	E	15	(TTA)29	50	3	0.61
24	SATT 187	A2	8	(TAA)18	54	2	0.45
25	SATT 193	F	13	(TAA)23	56.5	2	0.50
26	SATT 194	C1	4	(ATT)4GAGTAAATAG(TA)5	60	2	0.46
27	SATT 196	K	9	(TTA)5TTG(TTA)12(AGA)4	56	3	0.42
28	SATT 197	B1	11	(ATT)20	56.5	3	0.35
29	SATT 200	A1	5	(ATA)17	52	2	0.50
30	SATT 202	C2	6	(TTA)15	56.4	2	0.50
31	SATT 212	E	15	(TAA)9	53	2	0.20
32	SATT 220	M	7	(ATT)18ACCTTGGGA(TCC)4	55	2	0.44
33	SATT 229	L	19	(AAT)22	58	2	0.47
34	SATT 231	E	15	(TAT)32	61	2	0.26
35	SATT 236	A1	5	(ATT)19	63	2	0.50
36	SATT 238	L	19	(TTA)12	54	2	0.50
37	SATT 239	I	20	(AAT)22	61	2	0.21
38	SATT 242	K	9	(TTA)26	50	2	0.20
39	SATT 250	M	7	(TA)12	54	2	0.18
40	SATT 257	N	3	(ATA)10	60	3	0.64
41	SATT 270	I	20	(TTA)16	58.3	3	0.61
42	SATT 274	D1b	2	(TAT)18	61	2	0.21
43	SATT 277	C2	6	(ATA)41	58	2	0.04
44	SATT 286	C2	6	(ATT)18	56.8	2	0.06
45	SATT 294	C1	4	(TAT)23	60.2	2	0.50
46	SATT 302	F	12	(ATA)13AAG(TAA)4	55.9	2	0.49
47	SATT 303	G	18	(TAA)20	47.3	3	0.55
48	SATT 308	M	7	(TTA)22	62.5	4	0.72
49	SATT 313	L	19	(ATT)14	62	2	0.50

Table 5. Contd.

50	SATT 317	H	12	(TCAT)3(TTA)21	59	2	0.34
51	SATT 335	F	13	(TCT)4	57	3	0.46
52	SATT 342	D1a	1	(ATT)21	58.2	2	0.48
53	SATT 353	H	12	(TTA)17	63	2	0.50
54	SATT 355	E	15	(CAT)6(AAT)14	61	3	0.41
55	SATT 358	O	10	(ATA)19	63	3	0.46
56	SATT 371	C2	6	(TAA)11	56.2	2	0.40
57	SATT 384	E	15	(ATA)16	61	2	0.15
58	SATT 396	C1	4	(TTA)9	56	2	0.11
59	SATT 398	L	19	(ATTA)3	61	2	0.47
60	SATT 399	C1	4	(ATT)14	54.5	2	0.15
61	SATT 415	B1	11	(TAA)4	59.2	2	0.20
62	SATT 417	K	9	(AAT)18	54	2	0.49
63	SATT 420	O	10	(TAT)16	57	2	0.44
64	SATT 423	F	13	(TAT)19	50	2	0.47
65	SATT 426	B1	11	(ATT)5	62.7	2	0.13
66	SATT 434	H	12	(ATA)32	55.5	2	0.44
67	SATT 442	H	12	(TAA)35	61	2	0.40
68	SATT 449	A1	5	(TTA)21	56	3	0.63
69	SATT 458	D2	17	(TAT)30	64	2	0.50
70	SATT 468	D1a	1	(ATTT)3TGAAATTCTTCATATT(TTA)14	59	2	0.34
71	SATT 476	C1	4	(ATA)20	56.3	2	0.49
72	SATT 480	A2	8	(TAT)14	56.4	2	0.37
73	SATT 496	I	20	(ATT)13	62	2	0.50
74	SATT 510	F	13	(TAT)9	62	3	0.64
75	SATT 540	M	7	(TTA)15	58	2	0.49
76	SATT 542	D1b	2	(TAA)19	55	2	0.16
77	SATT 545	A1	5	(TTA)24	52	2	0.49
78	SATT 551	M	7	(AAT)8	54	2	0.33
79	SATT 556	B2	14	(AAT)14	54	3	0.56
80	SATT 562	I	20	(TTA)18	57	2	0.37
81	SATT 571	I	20	(ATA)14	50	3	0.52
82	SATT 591	A1	5	(ATT)17	50.8	2	0.26
83	SATT 610	G	18	(ATA)9	56.8	2	0.46
84	SATT 632	A2	8	(AAT)17	53.6	2	0.50
85	SATT 663	F	13	(TTA)27CTATTACTATTAC(TAT)4	56	2	0.21
86	SATT 703	D1b	2	(ATT)27	56.9	2	0.47
Total						195	
Mean						2.27	0.42

Table 6. Minimum and maximum measurements of dissimilarity obtained between cultivars belonging to the same genetic breeding programs.

Breeding	Minimum		Maximum	
Programs	Jaccard's Coefficient	Pairs	Jaccard's Coefficient	Pairs
Brasmax	0.0764	5-8	0.5102	4-6
Coodetec	0.2666	11-14	0.5617	10-14
Embrapa	0.1497	16-17	0.6104	16-23
Monsanto	0.3773	46-47	0.5901	39-47
Pioneer	0.1923	53-54	0.5577	54-57
TMG	0.2374	65-66	0.6031	68-73

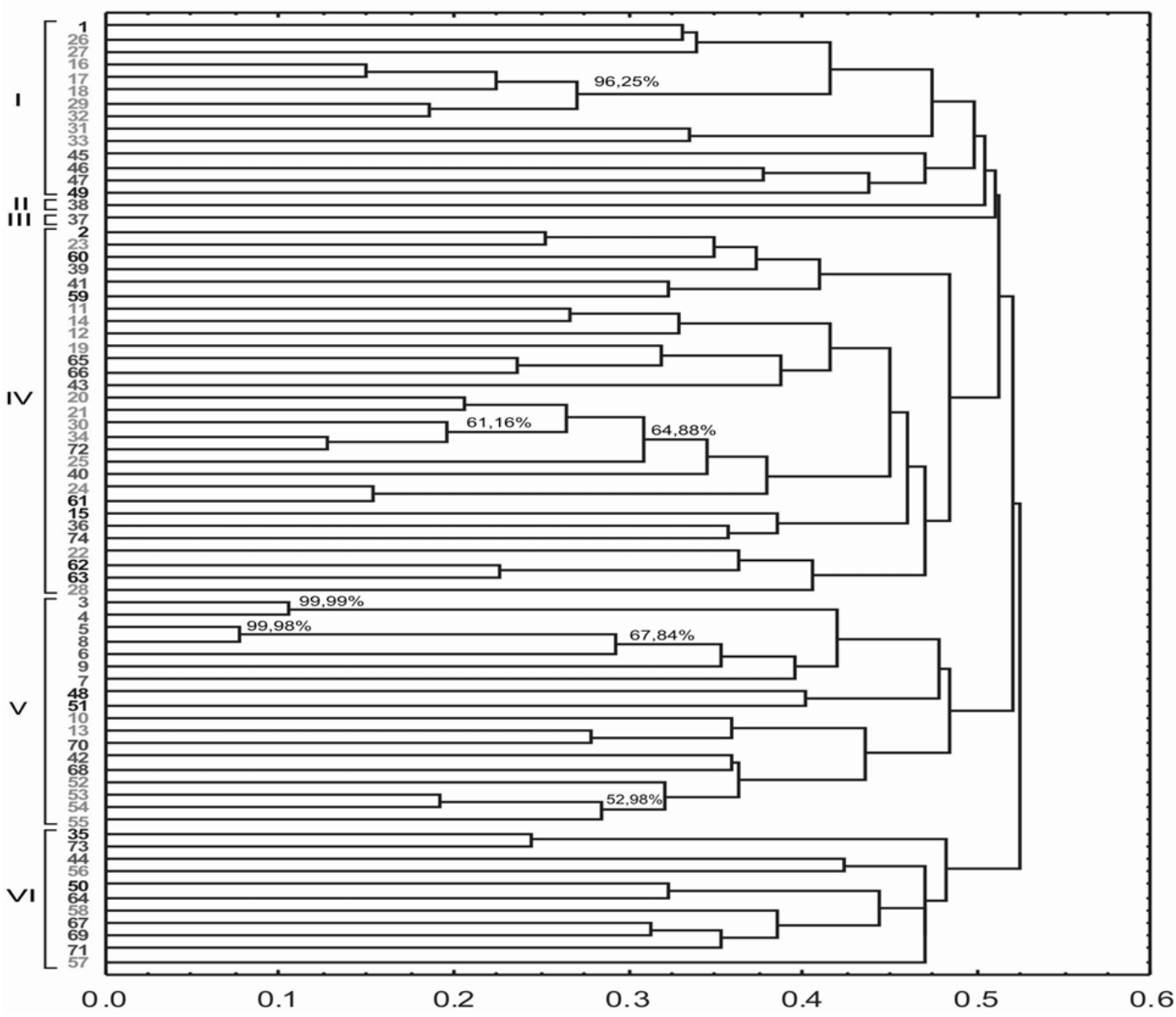


Figure 2. Dendrogram obtained using the UPGMA method, representing genetic dissimilarity among 74 RR soybean cultivars, based on 86 SSR markers. Bootstrap node support, represented in percentages, shows clustering stability.

program showed the lowest dissimilarity between cultivars (0.07), among all minimum measures observed. Moreover, it also had the lowest dissimilarity between cultivars (0.51) when compared to the maximum distances among programs, indicating the existence of lower genetic variability among their cultivars.

The UPGMA cluster analysis, based on the genetic dissimilarity matrix, showed that the 74 cultivars formed six major groups (Figure 2). Bootstrap analysis expressed high statistical support for the most part of the nodes in the dendrogram. The cophenetic correlation between the dissimilarity matrix and the dendrogram was significant at 1% of probability (0.66) by the test t. Bootstrap analysis and cophenetic correlations indicated that SSR dendrogram clustering accurately depicted estimated genetic distances among soybean cultivars. Group I contained 14 cultivars, which represented 18.9% of the total cultivars evaluated. The cultivars M7578 and M7211 formed the isolated groups II and III, respectively. Group IV was the largest group consisting of 29 genotypes (39.1%), including cultivars from almost all the breeding programs. Group V and VI were formed by contained 18 and 11 cultivars representing respectively 24.3 and 14.8% of the total genotypes evaluated. Analyzing cultivars distribution within breeding programs, the 12 genotypes from Monsanto were distributed in all the six groups formed (I, II, III, IV, V and VI) and two of them were in the isolated groups II (M7578) and III (M7211).

The 11 TMG cultivars were clustered in the groups IV, V and VI. The 19 cultivars from Embrapa showed less diversity due to their clustering in only two groups (I and IV), were the Coodetec and Pioneer cultivars, which were distributed in the groups IV and V and V and VI, respectively. All the Brasmax cultivars were clustered in the group V, indicating a greater genetic similarity between them.

Through the genealogy of some cultivars belonging to the same group, it is possible to verify parental in common, such as BRS243 and BRS244 from Embrapa. Both have genealogy Embrapa 59 and the bulk E96 246 as similar parental. The low genetic diversity found may be due to evaluation of sib lines. The dendrogram showed the formation of two subgroups exclusive to Embrapa and Brasmax breeding programs. The BRS 243, BRS 244, BRS 246, BRS Charrua and BRS Pampa cultivars from Embrapa formed a subgroup within the group I. Despite this clustering, the Embrapa cultivars were distributed into two groups, which indicated variability. The opposite occurred with the Brasmax cultivars (BMX Apolo, BMX Energia, BMX Força, BMX Potência, BMX Impacto and BMX Magna), all of them formed a subgroup within the group V, indicating a close similarity and practically no genetic variability in its germplasm. Cluster analysis using hierarchical methods have been widely used in studies of genetic diversity (Yamanaka et al., 2007; Singh et al., 2010). Wang et al. (2010) studying genetic variability in 40 soybean accessions of cultivars, landraces and wild soybeans collected from China found that wild soybeans and landraces possessed greater allelic diversity than cultivars and the UPGMA results also exhibited that wild soybean was of more abundant genetic diversity than cultivars.

Moreover, hierarchical methods have shown good agreement between the dendrograms generated and the kinship among accessions evaluated. Bonato et al. (2006) observed that the dendrogram obtained with AFLP markers was consistent with the pedigree of soybean genotypes analyzed. Priolli et al. (2010) found that the clustering of 168 soybean cultivars obtained by UPGMA method, based on the information of SSR markers, were consistent with ancestors which are common among cultivars within the same group.

Comparison between phenotypic and molecular analyses

Both the phenotypic data, represented by the agronomic traits, as the molecular data proved to be a useful tool on diversity characterization among the RR cultivars. Both methods demonstrated that Monsanto cultivars were clustered into various groups, indicating highest diversity among cultivars, which may be due to this company having been responsible for the development of RR soybeans and to their strong research effort in this area (Green, 2009). Although, the TMG cultivars have been grouped into fewer groups, they did show a relatively high genetic divergence when analyzed by both methods. Moreover, the methods equally indicated that the Coodetec, Pioneer and Brasmax programs had a low diversity since these genotypes were clustered in only one or two groups. The use of highly related genotypes as receptors of the RR gene within the soybean breeding programs may have caused the low genetic diversity observed in this study.

Li et al. (2008) observed that soybean cultivars from the same breeding programs were clustered in the same group and attributed this to a restricted use of parents in developing these cultivars. Vieira et al. (2009) also described low genetic diversity among cultivars from the same breeding program when they evaluated 53 soybean cultivars commercialized in Brazil. The formation of one group containing most of the 74 cultivars was also observed on the phenotypic and molecular analyses demonstrating the genetic similarity among RR soybean cultivars, even for cultivars belonging to distinct breeding programs. Santos et al. (2011), analyzing diversity between 48 Brazilian soybean cultivars, observed a tendency for transgenic cultivars to form a single and very similar group.

Sneller (2003), studying the genetic structure of soybean elite population in North America and the effect of recurring crosses with RR soybeans on the genetic divergence of these lines, concluded that RR technology generally had only a small impact on cultivar genetic diversity. However, based on the low diversity found between the elite lines of some companies, the author concluded that the low diversity in some programs, with the low germplasm exchange, could affect the available variability in the future. Studying the genetic structure basis of soybean in Brazil, Wysmierski and Vello, (2013) pointed out an increasing number of ancestors over all period, as well as its relative genetic contribution also increased from 46.6 to 57.6%, indicating a narrowing of the genetic base. These authors suggested if there is interest by the companies to increase the genetic base, they should choose the parents with the most divergent pedigrees. Contradicting these results, Vieira et al. (2009), Santos et al. (2011) and Peluzio et al. (2009), have detected variability among soybean elite populations in Brazil. Although both analyses shared most of the results, there were some differences. The most divergent cultivar pairs identified with Euclidean distance (phenotypic data) differed from those obtained with the Jaccard Coefficient (molecular data). However, it can be seen that for both methods, the minimum distances always occurred among the Brasmax cultivars. Differences were also observed in the cultivars clustering within the Embrapa program. Such cultivars were less divergent when analyzed by molecular markers being separated into two groups and not into six as occurred

with the phenotypic analysis.

The correlation coefficient between genetic distances estimated by phenotypic and molecular data was low but significant (r=0.11, P<0.01. t-Test and Mantel's Test with 10,000 simulations). Gouvêa et al. (2010) also observed low correlations between the genetic distances based on SSR and the phenotypic data in the rubber tree (r=0.13, P<0.01). However, Li et al. (2008) found moderate correlation coefficients (r=0.31 p < 0.01) in soybean utilizing SSR markers. Chiorato et al. (2007), on correlating matrices from agronomic variables and RAPD molecular descriptors in dry beans, also found moderate correlation coefficients (r=0.33, p<0.01). The difference between the most divergent cultivar pairs found from the Euclidean distance and Jaccard's Coefficient, as well as the low correlation between the phenotypic and molecular data, indicate that each method estimated the divergence between genotypes in a distinct way. According to Roldan-Ruiz et al. (2001), an alternative way to deal with the low correlation between genetic and phenotypic distance, would be selecting only molecular markers associated with phenotypic traits. Another factor which makes the occurrence of an association between phenotypic and molecular data more difficult to observe is that the variation detected by the molecular markers is not adaptive and, therefore, not subject to selection, in contrast to the agronomic traits, which are subject to both natural and artificial selection, as well as suffering a significant environmental influence (Vieira et al., 2005). The soybean cultivars used in this study are a representative sample of the RR cultivars grown and commercialized in Brazil. Therefore, it was possible to make an inference on the existing genetic diversity into the breeding programs that developed these cultivars. Even without the genealogical information, the dendrograms developed from the phenotypic and molecular data grouped cultivars according to their origins.

The results of this study show that some breeding programs had less genetic diversity, indicating the use of a narrow genetic base for developing their RR cultivars. The introduction of variability into soybean breeding programs to generate new combinations from the widening of the genetic base of this crop is fundamental for dealing with new demands and avoiding the risks of genetic vulnerability. The selection of more divergent cultivars, based on the dendrograms presented, is a viable alternative, which can be used commercially to avoid production losses related to the extensive use of cultivars with a narrow genetic base.

Conclusion

The existence of genetic variability between RR soybean cultivars was verified. Both agronomic traits and SSR molecular markers are useful tools for estimating the existing divergence among RR cultivars. Multivariate techniques based on agronomic traits and SSR molecular markers show differential ability to estimate genetic divergence between genotypes and should be used as complementary tools.

Conflict of Interests

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

REFERENCES

Arshad M, Naazar A, Ghafoor A (2006). Character correlation and path coefficient in soybean [*Glicine max* (L.)] Merill. Pak. J. Bot. 38(1):121-130.

Beyene Y, Botha AM, Myburg AA (2005). A comparative study of molecular and morphological methods of describing genetic relationships in traditional Ethiopian highland maize. Afr. J. Biotechnol. 4(7):586-595.

Bizari EH, Unêda-Trevisoli SH, Vianna VF, Meyer AS, Di Mauro AO (2014). Genetic diversity in early-maturing soybean genotypes based on biometric and molecular parameters. J. Food Agric. Environ. 12(1):259-265.

Bonato ALV, Calvo ES, Geraldi IO, Arias CAA (2006). Genetic similarity soybean (*Glycine max* L. Merrill) cultivars released in Brazil using AFLP markers. Gen. Mol. Biol. 29(4):692-704.

Carvalho LP, Lanza MA, Fallieri J, Santos JW (2003). Análise da diversidade genética entre acessos de banco ativo de germoplasma de algodão. Pesq. Agrop. Bras. 38(10):1149-1155.

Chiorato AF, Carbonell, SAM, Benchimol LL, Chiavegato, MB, Dias LA., Colombo CA (2007). Genetic diversity in common bean accessions evaluated by means of morpho-agronomical and RAPD data. Sci. Agric. 64(3):256-262.

Coelho AS, Bood G (2002). Avaliação dos erros associados a estimativas de distâncias/similaridades genéticas através do procedimento de bootstrap com número variado de marcadores (software). Goiânia: UFG, Instituto de Ciências Biológicas, Laboratório de Genética Vegetal, 2002.

Cruz CD (2008). Programa Genes - Diversidade Genética. Versão Windows. Viçosa: Editora UFV.

Cui Z, Carter TE, Burton JW, Wells R (2001). Phenotypic Diversity of Modern Chinese and North American Soybean Cultivars. Crop Sci. 41:1954-1967.

Ferreira ME, Grattapaglia D (1998).Introdução ao uso de marcadores moleculares em análise genética. 3nd ed. Brasília: EMBRAPA-CENARGEN. p. 220.

Fu YB, Peterson GW, Morrison MG (2007). Genetic diversity of Canada soybean cultivars and exotic germplasm revealed by simple repeat markers. Crop Sci. 47:1947-1954.

Gouvêa LRL, Rubiano LB, Chiorato AF, Zuchi MI, Gonçalves OS (2010). Genetic divergence of rubber tree estimated by multivariate techniques and microsatellite markers. Gen. Mol. Biol. 33(2):308-318.

Green JM (2009) .Evolution of glyphosate-resistant crop technology. Weed Sci. 57: 108-117.

Guan R, Chang R, Li Y, Wang L, Liu Z, Qiu L (2010). Genetic diversity comparison between Chinese and Japanese soybeans (Glycine max (L.) Merr.) revealed by nuclear SSRs. Genet. Resour. Crop. Evol. 57:229-242.

Hajjar R, Jarvis DI, Gemmill-Herren B (2008). The utility of crop genetic diversity in maintaining ecosystem services. Agric. Ecosyst. Environ. 123: 261–270.

Huang XQ, Wolf M, Ganal MW, Orford S, Koebner RMD, Röder MS (2007). Did Modern Plant Breeding Lead to Genetic Erosion in European Winter Wheat Varieties? Crop Sci. 47(1): 343-349.

James C (2013). Global Status of Commercialized Biotech/GM Crops: 2013. ISAAA Brief No. 46. ISAAA: Ithaca, NY.

Jose FC, Mohammed MM, Thomas G, Varghese G, Selvaraj N, Dorai M (2009). Genetic diversity and conservation of common bean (*Phaseolus vulgaris* L., Fabaceae) landraces in Nilgiris. Curr. Sci.

97(2): 227-235.
Kuroda Y, Tomooka N, Kaga A, Wanigadeva S, Vaughan D (2009). Genetic diversity of wild soybean (*Glycine soja* Sieb. et Zucc.) and Japanese cul- tivated soybeans [*Glycine max* (L.) Merr.] based on microsatellite (SSR) analysis and the selection of a core collection. Gen. Res. Crop Evol. 56:1045-1055.
Laborda PR, Oliveira KM, Garcia AAF, Paterniani, MEAGZ, Souza AP (2005). Tropical maize germplasm: What can we say about its genetic diversity in the light of molecular markers? Theor. Appl. Gen. 111:1288-1299.
Li W, Han Y, Zhang D, Yang M, Teng W, Jiang Z, Qiu L, Sun G (2008). Genetic diversity in soybean genotypes from north-eastern China and identification of candidate markers associated with maturity rating. Plant Breed. 127:494-500.
Liu M, Zhang M, Jiang W, Sun G, Zhao H, Hu S (2011). Genetic diversity of Shaanxi soybean landraces based on agronomic traits and SSR markers. Afr. J. Biotechnol. 10(24):4823-4837.
Malik MFA, Ashraf M, Qureshi AS, Gjafoor A (2007). Assessment of genetic variability, correlation and path analyses for yield and its components in soybean. Pak. J. Bot. 39:405-413.
Mikel MA, Diers BW, Nelson RL, Smith HH (2010). Genetic Diversity and Agronomic Improvement of North American Soybean Germplasm. Crop Sci. 50:1219-1229.
Miranda ZFS, Arias CAA, Prete CEC., Kihl RAS, Almeida LAA,Toledo JFF, Destro D (2007). Genetic characterization of ninety elite soybean cultivars using coefficient of parentage. Pesq. Agrop. Bras. 42(3): 363-369.
Mulato BM, Moller M, Zucchi MI, Quecini V, Pinheiro JB (2010). Genetic diversity in soybean germplasm identified by SSR and EST-SSR markers. Pesq. Agrop. Bras. 45(3):276-283.
Narjesi V, Khaneghah HZ, Zali EE (2007). Assesment of genetical relationship in few important agronomic characters with grain yield in soybean by multivariate ststistical analysis. Agric. Nat. Res. Sci. 41(11):227-234.
Peluzio JM, Pires LPM, Cancellier LL, Afférri FS, Colombo GA, Teixeira Júnior T, Ribeiro GRS (2012). Genetic divergence among soybean cultivars in irrigated lowland in the State of Tocantins. Ciência Rural 42(3):395-400.
Peluzio JM, Vaz-de-Melo A, Afférri FS, Silva RR, Barros HB, Nascimento IR, Fidelis RR (2009). Variabilidade genética entre cultivares de soja, sob diferentes condições edafoclimáticas. Pesq. Apli. Agrotec. 2(3):21-29.
Priolli RHG, Pinheiro JB, Zucchi MI, Bajay M, Vello NA (2010). Genetic Diversity among Brazilian soybean cultivars based on SSR loci and pedigree data. Braz. Arch. Biol. Technol. 53(3):519-531.
Reina E, Peluzio JM, Afferri FS, Oliveira Junior PW, Siebeneichler SC (2014). Genetic divergence and phosphorus use efficiency in the soybean with a view to biodiesel production. Rev. Cienc. Agron. 45(2) 344-350.
Roldan-Ruiz I, Euwijk FA, Gilliland TJ, Dubreuil P, Dillmann C, Lallemand J, Loose M, Baril CP (2001). A comparative study of molecular and morphological methods of describing relationships between perennial ryegrass (*Lolium perenne* L.) varieties. Theor. Appl. Gen. 103:1138-1150.
Salimi S (2013). Genetic diversity in soybean genotypes under water stress and normal condition using factor analysis and cluster analysis. Afr. J. Agric. Res. 8(49):6529-6536.
Salimi S, Lahiji SH, Abadi GM, Moradi S (2012). Genetic Diversity in Soybean Genotypes Under Drought Stress Condition Using Factor Analysis and Cluster Analysis. Word. Appl. Sc. J. 16 (4):474-478.
Santos ER, Barros HB, Ferraz EC, Capone A, Santos AF, Fidelis RR (2011). Divergência entre genótipos de soja, cultivados em várzea irrigada. Rev. Cer. 58(6):755-764.
Schuster I, Vieira ESN, Silva GJS, Franco FA, Marchioro VS (2009). Genetic variability in Brazilian wheat cultivars assessed by microsatellite markers. Gen. Mol. Biol. 32(3):557-563.
Shadakshari TV, Kalaimagal T, Senthil N, Boranayaka MB, Kambe Gowda R, Rajesha G (2011). Genetic diversity studies in soybean [*Glycine max* (L.) Merrill] based on morphological characters. Asian J. Bio Sci. 6(1):7-11.
Shi A, Chen P, Zhang B, Hou A (2010). Genetic diversity and association analysis of protein and oil content in food-grade soybeans from Asia and the United States. Plant Breed. 129:250-256.
Singh RK, Bhatia VS, Bhat KV, Mohapatra T, Singh NK, Bansal KC, Koundal KR (2010). SSR and AFLP based genetic diversity of soybean germplasm differing in photoperiod sensitivity. Genet. Mol. 33(2):319-324.
Sneller CH (2003). Impact of transgenic genotypes and subdivision on diversity within elite North American soybean. Crop Sci. 43:409-414.
Statsoft Inc (2004). STATISTICA (data analysis software system), version 7. www.statsoft.com.
Tantasawat P, Trongchuen J, Prajongjai T, Jenweerawat S, Chaowiset W (2011). SSR analysis of soybean (Glycine max (L.) Merr.) genetic relationship and variety identification in Thailand. AJCS 5(3):283-290.
Vieira EA, Carvalho FIF, Oliveira AC, Benin G, Zimmer PD, Silva JAG, Martins AF, Bertan E, Silva G O, Schmidt DAM (2005) . Comparação entre medidas de distância genealógica, morfológica e molecular em aveia em experimentos com e sem a aplicação de fungicida. Bragantia 64(1):51-60.
Vieira ESN, Schuster I, Silva SB, Oliveira MAR (2009). Variabilidade genética em cultivares de soja determinada com marcadores microssatélites em gel de agarose. Pesq. Agrop. Bras. 44(11):1460-1466.
Wang LX, Guan RX, Liu ZX, Chang RZ, Qiu LJ (2006). Genetic diversity of Chinese cultivated soybean n revealed by SSR markers. Crop Sci. 46:1032-1038.
Wang M, Li RZ, Yang WM, Du WJ (2010). Assessing the genetic diversity of cultivars and wild soybeans using SSR markers. Afr. J. Biotechnol. 9:4857-4866.
Wysmierski PT, Vello NA (2013). The genetic base of Brazilian soybean cultivars: evolution over time and breeding implications. Genet. Mol. Biol. 36(4):547-555.
Yamanaka N, Sato H, Yang Z, XU DH, Catelli LL, Binneck E.; Arias CAA, Abdelnoor RV, Nepomuceno AL (2007). Genetic relationships between Chinese, Japanese, and Brazilian soybean gene pools revealed by simple sequence repeat (SSR) markers. Gen. Mol. Biol. 30(1):85-88.

academicJournals

Vol. 13(26), pp. 2626-2636, 25 June, 2014
DOI: 10.5897/AJB2014.13772
Article Number: C2DF31645607
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Chemical analyses, antibacterial activity and genetic diversity assessment of some Egyptian *Citrus* spp. cultivars

Sameh H. Youseif*, Abeer El- Halwagi, H. A. Sayed and Hanaiya A. El-Itriby

National Gene Bank and Genetic Resources (NGBGR), Agricultural Research Center, Ministry of Agriculture and Land Reclamation, Giza, Egypt, P. O. Box, 12619.

Received 4 March, 2014; Accepted 9 June, 2014

Citrus species are among the most important fruit trees in the world and are considered as a major export product of Egypt. Forty-eight *Citrus* L. accessions representing six citrus groups (orange, mandarin, lemon, sour orange, grape fruit and pummelo) were collected. Chemical proprieties including pH, total acidity, total soluble solids and ascorbic acid of different fruit juices were determined. Eight accessions representing different citrus groups were screened for their antibacterial activity against five pathogenic bacteria (*Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Micrococcus* spp. and *Bacillus pumilus*). Lemon and lime accessions exhibited the highest antibacterial activity compared to the standard antibiotics (ampicillin and streptomycin). However, grapefruit and pummelo accessions showed no inhibitory effect. Inter-simple sequence repeats (ISSR) markers were used to study the genetic diversity and phylogenetic relationships among citrus accessions. The highest level of polymorphism (71%) was detected amongst lemon and lime accessions, whereas, the lowest percentage of polymorphism (18%) was identified within the sour orange group. The phylogenetic tree separated the varieties into discrete clusters according to their respective citrus group. Citrus groups were initially divided into two main clusters at 0.18 level of similarity. Lemon, lime, mandarin and sour orange were grouped in the first cluster, while sweet orange, grapefruit and pummelo were nested in the second cluster.

Key words: Citrus, genetic diversity, ISSR markers, chemical analyses, antibacterial.

INTRODUCTION

The genus *Citrus* L. (family *Rutaceae*; sub-family *Aurantioideae*) includes some of the principal fruit crops of worldwide importance such as the citrons (*C. medica* L.), lemons [*C. limon* (L.) Osbeck], limes [*C. aurantifolia* (Christm.) Swingle], mandarins (*C. reticulate* Blanco), sour oranges (*C. aurantium* L.), sweet oranges [*C. sinensis* (L.) Osbeck], grapefruits (*C. paradisi* Macf.) and pummelos [*C. maxima* (Burm.) Merr.] (Golein et al., 2012). Citrus fruits are recognized as an important component of the human diet, providing a variety of constituents important to human nutrition, including vitamin C (ascorbic acid), folic acid, potassium, flavonoids, coumarins, pectin and dietary fibers (Dugo and Di Giacomo, 2002). Flavonoids in citrus have a broad spectrum of

*Corresponding author. Email: Samehheikal@hotmail.com.

biological activities including antibacterial, antioxidant, antidiabetic, anticancer, analgesic, anti-inflammatory and antianxiety (Sidana et al., 2013).

Worldwide production of citrus reached more than 129 million tons from cultivated trees in 140 countries around the world FAOSTAT (2012). The statistics Division of the Food and Agriculture Organization of the \United Nations (FAO) http://faostat.fao.org/, making citrus the leading cultivated tree crop. Citrus production in the Mediterranean Basin (22,441 thousand tons) is third only to China and Brazil (FAO, 2012) and accounts for about 20% of the world citrus production and about 60% of the world fresh citrus trade (CLAM, 2007). Spain is the leading producing country, whereas Italy and Egypt rank second and third, respectively. Egypt represents about 15% of the total citrus production in the Mediterranean Basin (CLAM, 2007) and is considered the ninth largest citrus producer in the world (Ahmed, 2012) with a global market share of 3.1% of the world citrus production FAOSTAT (2012) The statistics Division of the food and Agriculture Organization of the United Nations (FAO) http://faostat.fao.org/. The production is mainly composed of oranges, mandarins and limes, which represent more than 98.8% of the total citrus area (Eid and Guindy, 2008).

Evaluation of genetic diversity and genetic relationships among various accessions is of fundamental importance for plant breeding programs. This information can provide predictive estimates of genetic variation within a species, thus facilitating breeding material selection (Qi et al., 2008). In recent years, the progress made in the development of DNA based marker systems has advanced our understanding of genetic resources diversity and their gene mapping (Kalia et al., 2011). Many citrus genetic maps have been developed over the past decade (Chen et al., 2007; Roose, 2007); each genetic map has a different mapping population type and size, genome coverage, and marker systems. Most of these maps were covered by a majority of randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), inter-simple sequence repeats (ISSR), and simple sequence repeats (SSR) markers (Gulsen et al., 2010). Among these markers, ISSR has been widely used to assess the genetic diversity between different citrus species (Sankar and Moore, 2001; Uzun et al., 2010; Yang et al., 2010).

The objectives of this study were to analyze the chemical properties of forty-eight citrus accessions, explore their genetic diversity at intra-and interspecific levels of variation and screen the antibacterial activity of a representative sample from each citrus group against human pathogenic bacteria.

MATERIALS AND METHODS

Plant materials

Samples from young leaves and fresh fruits were collected from 48 individual trees, representing six citrus groups (sweet orange, mandarin, lemon, lime, sour orange, grape fruit and pummelo) from Moshtohor on-farm collection, Faculty of Agriculture, Benha University (Table 1)

Preparation of juice

At the time of maturation and ripeness, ten fresh fruits for each accession were sampled. Fruits were washed in running tap water in the laboratory, surface sterilized with 70% alcohol, rinsed with sterile distilled water and cut open with a sterile knife and the juice pressed out into a sterile universal container. Then juice was filtered using 0.45 membrane filter (Millipore©, USA) into another sterile container to remove the seeds and other tissues.

Determination of pH and total acidity

Total acidity of the juices was determined by titration method as reported by Rekha et al., (2012). Fruit juice was diluted to 10% with distilled water and then titrated against 0.1N NaOH (standardized using standard Oxalic acid) using Phenolphthalein indicator. The end point was noted when the color changed from colorless to pale pink. All measures were done in triplicate and dilution factor was considered; total acidity was calculated in terms of citric acid using the following formula, Acidity (g/100 mL) = Normality of the juice x Equivalent weight of citric acid. The pH of citrus juice was determined using a digital pH meter (Thermo©, USA).

Determination of total soluble solids (TSS)

Total soluble solids (TSS) were measured using digital refractometer (Atago Co., Ltd., Tokyo, Japan). All measures were done in triplicate; the TSS results were reported as ° Brix.

Estimation of ascorbic acid (vitamin C) content

Ascorbic acid content in fruit juice was determined by the 2, 6 dichlorophenol-indophenol titrimetric method according to AOAC method No. 967.21 (AOAC, 2000). All measures were done in triplicate; the vitamin C content was expressed as mg/100 ml.

Bacterial strains and cultural conditions

The antibacterial tests were carried out against five human pathogenic bacteria; *E. coli*, *S. typhi*, *S. aureus*, *Micrococcus* spp., *B. pumilus*. The bacterial cultures were supplied from Microbiology Department, National Organization of Drug Control and Research, Giza, Egypt. The cultures were maintained on nutrient agar slants and sub-cultured for 24 h before use.

Antibacterial activity test

The agar diffusion method (CLSI, 2002; Prescott et al., 2002) was used as a preliminary assay for testing the antibacterial effect of crude juice extracts of eight citrus accessions (Valencia orange, Balady mandarin, Eureka and Rough lemon, Balady lime, Balady sour orange, Duncan grapefruit and Egyptian pummelo) that represent different citrus groups. A previously liquefied and sterilized nutrient agar medium (20 ml) was poured into Petri-plates of 100 mm size (to make uniform thickness) and kept for solidifying. One milliliter of 10^9 a log phase bacterial culture was spread over the solidified media. Wells of 10 mm diameter were made in each

Table 1. List of citrus accessions used in this study.

No.	NGB* accession	Common name	Scientific name	No	NGB* accession	Common name	Scientific name
Sweet orange				**Lemon and Lime**			
1	127	San Gwen	*C. sinensis*	27	153	Eureka	*C. limon*
2	128	Balady	*C. sinensis*	28	154	Eureka	*C. limon*
3	129	Jaffa	*C. sinensis*	29	155	Variegated Pink	*C. limon*
4	130	Regular bearing	*C. sinensis*	30	156	Variegated Pink	*C. limon*
5	131	Succari	*C. sinensis*	31	157	Sweet lemon	*C. limetta*
6	132	Mouzambique	*C. sinensis*	32	158	Sweet lime	*C. limetta*
7	133	Blood Balady	*C. sinensis*	33	159	Sweet lime	*C. limetta*
8	134	Tunisi	*C. sinensis*	34	160	Succari lime	*C. limetta*
9	135	Navel	*C. sinensis*	35	161	Rough lemon	*C. jambhiri*
10	136	Khalili White	*C. sinensis*	36	162	Balady lime	*C. aurantifolia*
11	137	Khalili Red	*C. sinensis*	**Sour orange**			
12	138	Greek compressed	*C. sinensis*	37	163	Balady	*C. aurantium*
13	139	Tanneriffe	*C. sinensis*	38	164	Balady	*C. aurantium*
14	140	Centrial	*C. sinensis*	39	165	Barzi	*C. aurantium*
15	141	Mezazie	*C. sinensis*	**Grapefruit**			
16	142	Mafred	*C. sinensis*	40	166	Grapefruit	*C. paradisi*
17	143	Roja	*C. sinensis*	41	167	White Grapefruit	*C. paradisi*
18	144	Valencia	*C. sinensis*	42	168	Duncan	*C. paradisi*
19	145	Golden Nagen	*C. sinensis*	43	169	Marsh	*C. paradisi*
20	146	Hamlin	*C. sinensis*	**Pummelo**			
Mandarin				44	170	Broad leaf	*C. maxima*
21	147	Cleopatra	*C. reshni*	45	171	Egyptian	*C. maxima*
22	148	Clementine	*C. reticulata*	46	172	Rabehe	*C. maxima*
23	149	Clementine	*C. reticulata*	47	173	Gizawe	*C. maxima*
24	150	Balady	*C. deliciosa*	48	174	Moneybi	*C. maxima*
25	151	Satsuma	*C. unshiu*				
26	152	Santara	*Citrus spp*				

*NGB: National gene Bank, Giza, Egypt.

plate with a sterilized stainless steel borer. One hundred µl of fresh juice sample was poured into the well and compared to 100 µl Ampicillin and Streptomycin antibiotics (100 µg/ml) (Serva©, Germany) as standards. Sterilized H_2o was used as a negative control. The plates were performed in triplicates and then left standing for 3 hrs at 4°C (Sultana et al., 2012) for proper diffusion of the tested juices. After diffusion process, all the Petri plates were incubated at 37°C for 24 h, and then they were observed for zones of inhibition.

DNA extraction and ISSR analysis

Total DNA extraction was isolated using DNeasy Plant Mini Kit (Qiagen©, Germany) according to the manual procedures. Out of 30 tested ISSR primers, a total of 13 primers (Table 2), that generated clear reproducible banding patterns, were chosen for the final analysis. PCR reaction was performed in 25 µl reaction mix containing 1 X PCR buffer, 2 mM $MgC1_2$, 0.2 mM of each dNTPs, 1 µM oligonucleotide primer, 25 ng genomic DNA and 1 unit of Taq DNA polymerase (Promega©, USA). Amplification was performed in a 96-well Thermal Cycler (BioRad©, USA) under the following conditions: 3 min at 94°C for 1 cycle, followed by 1 min at 94°C, 1 min at annealing temperature (Table 2) , and 2 min at 72°C for 35 cycles, and 7 min at 72°C for a final extension step. PCR products were separated by electrophoresis on a 1.5% agarose gel stained by ethidium bromide and photographed by gel documentation (BioRad©, USA). The banding patterns generated by ISSR markers were analyzed and compared to determine the genetic diversity and relatedness among different citrus genotypes. The amplified fragments were scored either as present (1) or absent (0). The genetic similarity and similarity matrix among genotypes were estimated according to Dice coefficient (Sneath and Sokal, 1973) and based on Nei's (1972) genetic distance. Dendrograms showing phylogenetic relationships were constructed using the Un-weighted Pair Group Method with Arithmetic Averages (UPGMA) by Phoretix 1D software (TotalLab, UK).

Statistical analysis

The physiochemical data were statistically analyzed using SPSS Software for Windows (version 21; SPSS Inc., USA) to evaluate the significant differences at $p<0.05$ and to construct the correlation matrix. Antibacterial activity data were analyzed using the MSTATC analysis software according to Snedecor and Cochran (1980).

Table 2. List of primer names, sequences, annealing temperatures, total number, and size of amplicons and number of polymorphic bands as revealed by ISSR markers among the 48 citrus accession.

Primer	Sequence	Annealing temperature (°C)	PCR amplified fragments		Number of polymorphic bands
			Number	size range (bp)	
H_{12}	$(GA)_8YT$	41.0	19	140-1300	19
H_{13}	$(GA)_8YC$	42.5	22	90-2434	22
H_{14}	$(GA)_8YG$	44.0	20	180-1160	20
H_{15}	$AG)_8YT$	52.0	21	180-1510	20
H_{16}	$(AG)_8YC$	56.5	26	100-2530	26
H_{17}	$(AG)_8YG$	59.5	24	185-1466	24
H_{21}	$(GT)_8YC$	60.5	22	180-3470	22
H_{29}	$(GACA)_4AT$	41.5	19	195-1700	19
P_2	$(CA)_6GG$	48.0	25	180-1500	25
P_3	$(CA)_6AC$	42.5	21	195-1425	21
P_4	$(GTG)_3GC$	52.5	23	180-1360	23
P_{11}	$(GAG)_3GC$	45.0	17	220-1280	17
P_{16}	$ACG(GT)_7$	50.0	19	190-810	18
Total	-	-	278	-	275
Average	-	-	21.4	-	21.2

RESULTS

Chemical analysis of citrus fruit juices

Some chemical properties including pH, total acidity, total soluble solids and ascorbic acid of fresh fruit juices are shown in Table 3. The results show that, pH ranged from 2.50 in variegated pink lemon (Accessions NGB 155 and NGB-156) to 6.79 in Tunisi sweet orange fruits (NGB-134). However, titratable acidity ranged from 0.40% in Sweet lemon (NGB-157) fruits to 8.82 g citrate/100 ml in Balady sour orange (NGB-164). The highest solid soluble content (12.2^{o}Brix) was determined in Cleopatra mandarin (NGB-147), while the lowest soluble solids content (5.9°Brix) was detected in Variegated pink lemon (NGB-155 and NGB-156). The ascorbic acid content ranged from 4.2 mg/100 ml in Sweet lemon (NGB-157) to 56.9 mg/100 ml in Hamlin sweet orange (NGB-146) and Egyptian pummelo (NGB-171).

Antibacterial screening of citrus juice

The antibacterial activity of fresh juice of eight accessions representing different citrus groups compared to standard antibiotics (Ampicillin and Streptomycin) was screened against five pathogenic bacteria using agar diffusion assay (Table 4). Lemon and lime accessions exhibited the highest antibacterial activity against tested pathogenic bacteria, followed by Balady mandarin (NGB-150) which showed a moderate activity. Whereas, Valencia sweet orange (NGB-144) and Balady sour orange (NGB 163) displayed a feeble bioactivity. On the other hand, Duncan grapefruit and Egyptian pummelo accessions had no inhibitory effect. In general, Balady lime (NGB-162) had the highest antibacterial activity compared to other tested *Citrus* species (Table 4 and Figure 1).

Genetic diversity within *Citrus* genus

The genetic diversity of 48 citrus accessions was studied using 13 selected ISSR primers (Table 2) which generated reproducible and scorable patterns, compared to the other primers, which produced smears, or fuzzy patterns that could not be scored. Out of the 278 total amplified amplicons, 275 were polymorphic. The number of fragments amplified per primer varied from 17 (primer P_{11}) to 26 (Primer H_{16}), with an average of 21.4 fragments per primer (Table 2).

The highest number of amplified polymorphic amplicons was identified among lemon and lime group, which resulted in 71% polymorphism (Table S1). However, the lowest number of amplified polymorphic amplicons was detected among sour orange group, which resulted in 18% polymorphism (Table S1).

Genetic differentiation between species

The analysis of amplified amplicons produced by ISSR primers revealed the presence of positive unique markers that allowed the differentiation between different citrus species (Table S2). Six *Citrus* species (*C. sinensis*, *Citrus* spp., *C. limetta*, *C. jambhiri, C. aurantifolia* and *C. aurantium*) were discriminated by species specific unique ISSR markers (Table S2). The number of unique markers ranged from one in *C. limetta* species to 33 in *C. aurantium* species.

Table 3. Total soluble solids (TSS), pH, acidity percent and ascorbic acid of citrus fruits juice*.

Variety	Citrus Acces.	TSS Brix°		pH		Acidity gm citrate/100 ml		Ascorbic acid**	
	127	ND		ND		ND		ND	
	128	8.7	± 0.12	3.97	± 0.02	2.00	± 0.07	35.0	± 1.6
	129	9.1	± 0.10	4.09	± 0.00	1.84	± 0.07	35.3	± 0.46
	130	8.8	± 0.06	3.97	± 0.00	1.52	± 0.07	43.7	± 0.46
	131	11.0	± 0.00	6.28	± 0.00	0.76	± 0.07	21.1	± 0.46
	132	9.6	± 0.06	3.77	± 0.02	2.56	± 0.07	53.7	± 2.09
	133	9.7	± 0.06	3.94	±0.04	2.16	± 0.12	37.4	± 1.82
	134	11.2	± 0.20	6.79	± 0.00	0.76	± 0.07	28.4	± 0.00
	135	11.6	± 0.06	4.36	± 0.01	1.16	± 0.07	38.9	± 0.91
Sweet	136	10.7	± 0.11	3.99	± 0.03	2.12	± 0.07	33.4	± 0.46
orange	137	9.2	± 0.00	3.97	± 0.00	1.52	± 0.14	41.6	± 0.91
	138	8.8	± 0.00	4.16	± 0.02	1.56	± 0.00	42.9	± 1.20
	139	12.1	±0.06	4.08	± 0.00	1.20	± 0.00	26.3	± 1.20
	140	10.2	± 0.06	4.20	± 0.01	1.96	± 0.07	37.7	± 1.20
	141	11.7	± 0.12	4.17	± 0.01	1.40	± 0.07	27.1	± 1.20
	142	9.8	± 0.06	3.99	± 0.02	2.64	± 0.12	41.3	± 1.64
	143	11.3	± 0.12	3.75	± 0.04	2.40	± 0.00	45.0	± 0.00
	144	10.3	± 0.12	3.53	± 0.03	3.85	± 0.12	38.2	± 0.91
	145	10.4	± 0.06	3.87	± 0.01	2.24	± 0.18	37.4	± 0.46
	146	10.8	± 0.00	4.11	± 0.00	1.24	± 0.07	56.9	±0.00
	147	12.2	± 0.06	3.70	± 0.00	0.78	± 0.01	16.8	± 0.00
	148	10.7	± 0.00	3.77	± 0.07	0.75	± 0.00	23.5	± 0.01
Mandarin	149								
	150	10.0	± 0.06	3.41	± 0.02	2.08	± 0.07	4.3	± 0.40
	151	9.1	± 0.00	3.59	± 0.13	0.68	± 0.00	14.2	± 0.06
	152	11.5	± 0.7	3.20	± 0.17	1.22	± 0.00	29.3	± 0.06
	153	8.1	± 0.00	2.82	± 0.01	6.46	± 0.07	22.9	± 0.79
	154								
	155	5.9	± 0.06	2.50	± 0.00	4.90	± 0.07	22.7	± 0.00
	156								
Lemon	157	7.1	± 0.12	5.94	± 0.01	0.40	± 0.07	4.2	± 0.46
and lime	158	10.1	± 0.06	5.91	± 0.04	0.84	± 0.12	5.5	± 0.79
	159								
	160	ND	ND	ND	ND				
	161	11.0	± 0.06	2.72	± 0.03	6.55	± 0.10	22.1	± 0.00
	162	12.0	± 0.00	2.68	± 0.00	5.17	± 0.00	26.3	± 0.46
	163	10.9	± 0.06	2.97	± 0.01	7.26	± 0.07	42.7	± 2.09
Sour	164	10.1	± 0.06	3.20	± 0.01	8.82	± 0.07	29.5	± 1.82
orange	165	9.0	± 0.00	2.98	± 0.00	7.70	± 0.12	21.3	± 0.79
	166	ND		ND		ND		ND	
Grapefruit	167	ND		ND		ND		ND	
	168	8.7	± 0.06	3.41	± 0.00	3.29	± 0.07	39.8	± 0.91
	169	9.8	± 0.06	3.37	± 0.02	3.13	± 0.00	43.2	± 1.64
	170	11.0	± 0.06	3.28	± 0.03	4.29	± 0.07	54.2	± 0.46
	171	11.0	± 0.06	3.39	± 0.02	4.25	± 0.07	56.9	± 1.37
Pummelo	172	9.9	± 0.06	3.52	± 0.00	1.56	± 0.05	19.2	± 0.00
	173	9.9	± 0.06	3.55	± 0.00	1.37	± 0.05	22.2	± 0.00
	174	9.0	± 0.00	2.85	± 0.00	4.83	± 0.18	27.8	± 0.01

ND, Not Determined; *Means are followed by standard deviation, ** Ascorbic acid was determined as mg/ 100 ml.

Table 4. Antibacterial activity of fresh juice of some citrus accessions compared to standard antibiotics (Ampicillin and Streptomycin) against tested pathogenic bacteria.

Tested citrus juice/ antibiotic	Variety	Zone of Inhibition (mm)*									
		E. coli		*S. typhi*		*S. aureus*		*Micrococcus* spp.		*B. pumilus*	
Valencia (144)	Sweet orange	2	± 0.58	3	± 0.58	4	± 0.58	3	± 0.58	2	± 0.58
Balady (150)	Mandarin	16	± 0.10	18	± 0.58	16	± 0.58	21	± 0.58	18	± 0.58
Eureka (NGB 153)	Lemon	18	± 0.58	24	± 1.00	28	± 1.53	26	± 1.53	20	± 1.53
Rough (NGB 161)	Lemon	18	± 1.15	22	± 1.00	25	± 1.73	29	± 3.2	23	± 3.51
Balady (NGB 162)	Lime	30	± 1.53	29	± 3.00	25	± 1.00	26	± 1.53	25	± 1.00
Balady (NGB 163)	sour orange	3	± 0.58	4	± 0.58	3	± 0.58	5	± 0.58	4	± 0.58
Duncan (NGB 168)	Grapefruit	-	-	-	-	-	-	-	-	-	-
Egyptian (NGB 171)	Pummelo	-	-	-	-	-	-	-	-	-	-
Ampicillin (100 µg/ ml)		25	± 1.15	26	± 0.58	20	± 1.53	31	± 1.00	25	± 1.73
Streptomycin (100 µg/ ml)		20	± 1.00	15	± 1.00	19	± 0.58	22	± 0.58	20	± 3.00

*Means followed by the Standard Deviation (SD)

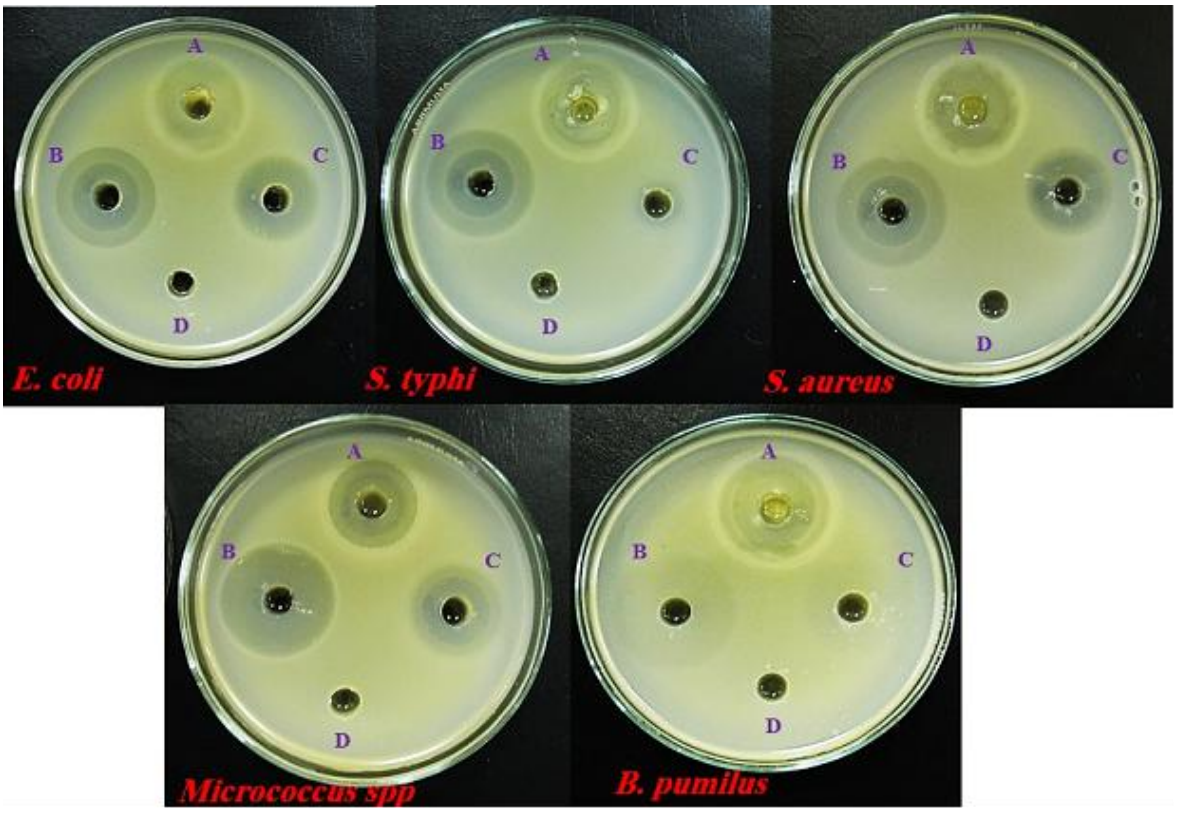


Figure 1. Antibiograms of Balady lemon juice (NGB-162) and used antibiotics (100 µg/ml) against tested bacteria. A, Balady lemon juice (NGB-162) juice; B, Ampicillin; C, Streptomycin; D, Sterile water.

Cluster analysis

Similarity matrix based on the ISSR data was calculated according to Dice coefficient (Sneath and Sokal, 1973). The highest genetic similarity (0.98) was identified among the accessions belonging to sweet orange group. However, the highest genetic diversity (0.12 similarity coefficient) was detected between lemon and lime group and sweet orange group. The UPGMA dendrogram based on the 278 ISSR amplified bands of the 48 accessions

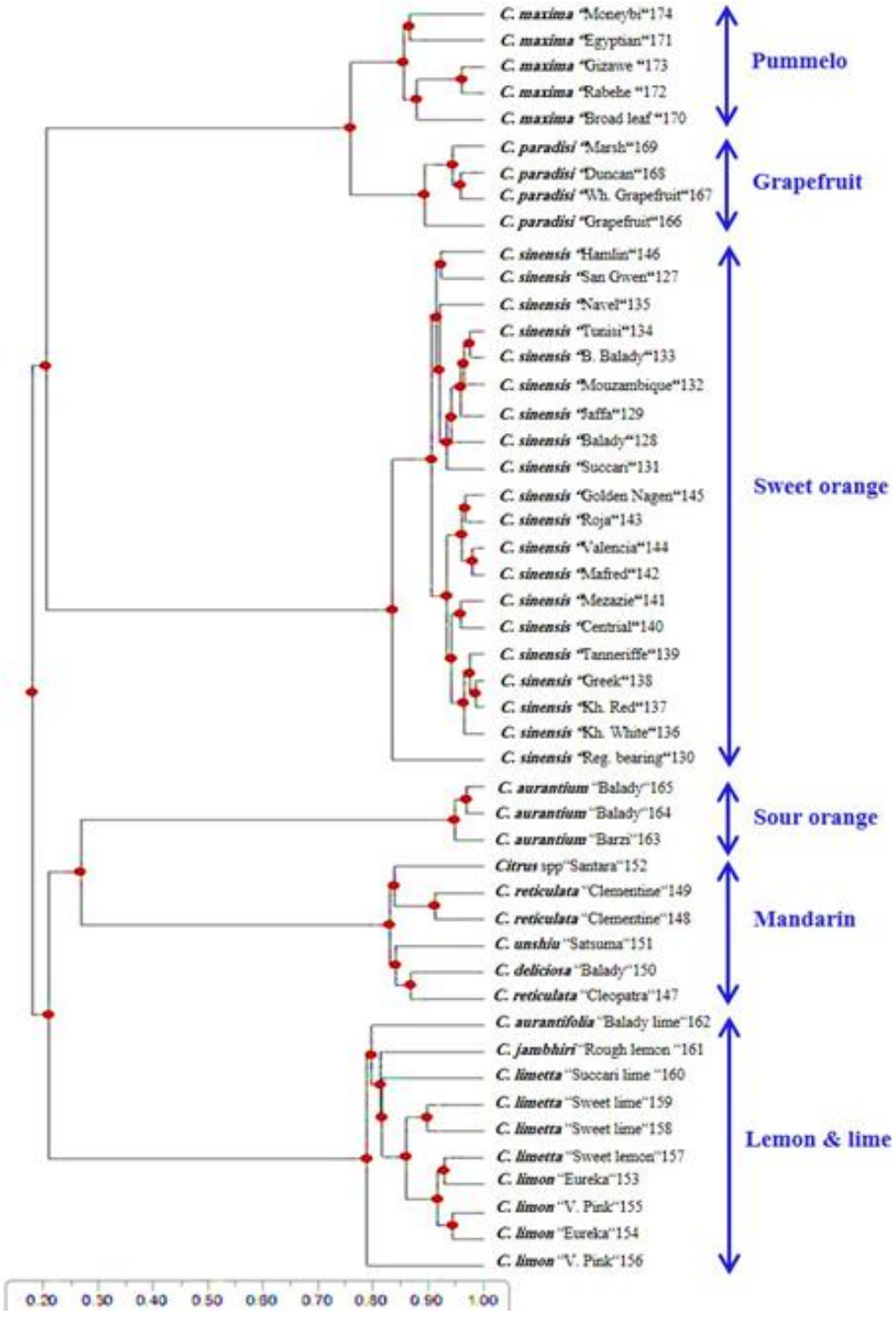


Figure 2. UPGMA dendrogram based on Nei's (1972) genetic distance for the forty-eight citrus accessions constructed by cluster analysis of ISSR markers. The numbers following the *Citrus* species are the accessions numbers.

(Figure 2), showed six well-defined lineages, corresponding to the different citrus groups (lemon, mandarin, sour orange, sweet orange, grapefruit and pummelo). The phylogenetic tree (Figure 2) divided citrus genotypes into two main clusters at 0.18 level of similarity; the first cluster included three sub-lineages; lemon, mandarin and sour orange groups, while the second cluster comprised sweet orange, grapefruit and pummelo groups.

DISCUSSION

Chemical properties of citrus fruits juice

Chemical composition of genetic resources is an essential identification process in monitoring of the genetic quality during improvement and conservation (IPGRI, 1999). Citrus is a good source of vitamin C, which is the most important nutrient component in citrus fruit juice (Xu et al., 2008). Our study showed that sweet oranges and pummelo fruits are good sources of vitamin C. These findings are compatible with other results published by other workers (Pichaiyongvongdee and Haruenkot, 2009; Hashempour et al., 2013). On the other hand, lemon and lime varieties showed a moderate ascorbic acid content (4-26 g/100 ml), which is in agreement with results reported by Rekha et al. (2012).

Antibacterial activity

Citrus varieties are considered a rich source of secondary metabolites which have the ability to produce a broad spectrum of biological activities (Johann et al., 2007; Ghasemi et al., 2009). Results of the current study showed a promising antibacterial activity of selected citrus fruit juice against some human pathogenic bacteria (*E. coli*, *S. typhi*, *S. aureus*, *Micrococcus* spp. and *B. pumilus*). Among the tested accessions (Valencia orange, Balady mandarin, Eureka and Rough lemon, Balady lime, Balady sour orange, Duncan grapefruit and Egyptian pummelo), that represent different citrus groups, lime and lemon juices exhibited the highest biological activity. Lemons and limes have been known as an important medicinal plants and the potentiality of their juices as antimicrobial agents was previously confirmed (Tomotake et al., 2006; Jayana et al., 2010; Bansode and Chavan, 2012; Hindi and Chabuck, 2013).

In general, antimicrobial activity of Citrus may be referred to their rich content of flavonoids. Citrus flavonoids have a large spectrum of biological activity including antibacterial, antifungal, antidiabetic, anticancer and antiviral activities (Burt, 2004). Lemon juice is characterized by the presence of significant amounts of the flavones, flavanones, hesperidin and eriocitrin (Gattuso et al., 2007). The bioactivity of lemon juice containing-flavonoids like luteolin and apigenin (Gattuso et al., 2007) have been previously reported (Cushnie and Lamb 2005).

Genetic analysis of the *Citrus* genus

Understanding the genetic variation within and among populations is essential for the establishment of effective and efficient conservation practices for plant genetic resources (Yang et al., 2010). ISSR markers technique has been known as a rapid, reproducible and useful method for distinguishing among different cultivars and clustering genotypes in the citrus species (Siragusa et al., 2006; Yang et al., 2010). In the present study, 13 primers produced clear, species-specific fingerprint patterns with all samples and were sufficient to discriminate varietal groups of citrus and produced results consistent with previous studies (Yang et al., 2010). Polymorphism analysis exhibited an average of 99.2% polymorphism among the forty-eight accessions under study. Similarly, Hussein et al., (2003) reported an average of 82.4% polymorphism generated by eight ISSR primers among fourteen Egyptian citrus genotypes. The UPGMA phylogenetic tree (Figure 2) clearly splits the Egyptian accessions into two main clusters. The first cluster included lemon, lime, mandarin and sour orange varieties. This finding is compatible with the hypothesis that, lemon originated from citron and sour orange, with sour orange being the maternal parent (Nicolosi et al., 2000; Gulsen and Roose, 2001). Also, clustering of mandarins and sour oranges into two sub-clusters, which is in agreement with the suggestion that mandarin, is the paternal parent of sour orange (Li et al., 2010). On the other hand, the second cluster included sweet orange, grapefruit and pummelo accessions. This grouping is in accordance with the hypothesis that pummelo is the maternal parent of sweet oranges (Nicolosi et al., 2000; Froelicher et al., 2011). Our study showed that, grapefruits were much closer to pummelos (0.76 genetic similarity) than sweet oranges (0.20 genetic similarity), which could confirm that grapefruit was derived from a backcross with pummelo (Barkley et al., 2006; Pang et al., 2007). In conclusion, the phylogenetic tree based on ISSR markers, separated the citrus varieties into discrete clusters according to their respective citrus group.

Intra- variation within each citrus group

Lemons and Limes

Lemons and limes account for 10.3 and 0.21% of the total citrus cultivated area in Egypt, respectively (Eid and Guindy, 2008). All the accessions were grouped in one lineage; however, the variegated pink lemon accession (NGB-156) was located separately from the other varieties exhibiting a 0.79 genetic similarity. The high genetic similarity found amongst lemons and limes was previously reported by other workers (Federici et al., 1998; Nicolosi et al., 2000). This genetic overlapping could be referred to the suggestion that, (*C. medica*) an ancestral of citrus species gave rise to lemons, limes, and rough lemons through various hybridization events (Barkley et al., 2006).

Mandarins

Mandarins account for 26.4% of the total citrus area in

Egypt. In the present study, a high degree of genetic similarity ranging from 0.83 to 0.90 was detected between the six mandarin accessions, although they belonged to four different species (*C*. *reticulate*, *C*. *delciosa*, *C*. *unshiu* and *Citrus* spp.). The phylogenetic dendrogram showed that, mandarins were closer to sour oranges than lemon and lime cultivars, which is in agreement with other results published by EL-Mouei et al., (2011).

Sour oranges

Sour orange is the most widely used citrus root stocks in Egypt (Eid and Guindy, 2008) and worldwide (Siraguse et al., 2006). A high genetic similarity coefficient ranging from 0.95 to 0.97 was detected among the three sour orange accessions. Similarly, Hussein et al., (2003) reported a high genetic similarity among Spanish, Balady and Brazilian sour orange accessions belonging to *C. aurantium* species.

Sweet oranges

Orange production accounts for about 61% of total citrus production in Egypt (Eid and Guindy, 2008). Three principal varieties of oranges are produced in Egypt; Navel (35%), Valencia (18.4%) and Baladi (7.0%). ISSR markers revealed a high level of genetic similarity ranging from 0.83 to 0.98 among the twenty orange accessions. This narrow genetic base among the sweet orange accessions has been previously reported in many publications (Fang and Roose, 1997; Targon et al., 2000; Snoussi et al., 2012).

Grapefruits and Pummelos

Grapefruit is the fourth economically most important citrus fruit in the world (Uzun et al., 2010). A high level of genetic similarity was detected among grapefruit accessions ranging from 0.89 to 0.97; this narrow genetic base among the grapefruit cultivars has been reported in previous publications using different molecular markers (Fang and Roose, 1997; Corazza-Nunes et al., 2002). The high level of similarity within the grapefruit group supported the hypothesis that the majority of grapefruit cultivars were derived from the same ancestral tree by mutations (Gmitter, 1995). Pummelo has played an important role as a parent of many citrus fruits, such as lemons, oranges and grapefruits. Among the five pummelo accessions, the genetic similarity ranged from a 0.87 to 0.97. This in line with other published reports (Corazza-Nunes et al., 2002; Uzun et al., 2010).

In conclusion, our study reveals the antibacterial activity of natural lemon and lime juices against human pathogenic bacteria, nevertheless further studies are needed to identify the chemical composition of different bioactive compounds containing juice and declare their relation to their antibacterial properties. Our study also, indicated the presence of high genetic diversity among different *Citrus* species and groups currently cultivated in Egypt, however a high level of genetic similarity was detected within each citrus group.

Conflict of Interests

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

ACKNOWLEDGEMENTS

The authors acknowledge FAO for providing funds for the molecular characterization research

REFERENCES

Ahmed HF (2012). Phytohormones content and random amplified polymorphic DNA (RAPD) marker assessment of some Egyptian citrus cultivars. Afr. J. Biotechnol. 11: 15755-15762.

AOAC, Association of Official Agricultural Chemists (2000) Official Method of Analysis. 17th Ed. Association of Official Analytical Chemists (No. 967.21 Ascorbic acid in vitamin preparation and juice), Gaithersburg, MD, USA.

Bansode DS, Chavan MD (2012). Studies on antimicrobial activity and phytochemical analysis of citrus fruit juices against selected enteric pathogens. Int. Res. J. Pharm. 3:122-126.

Barkley N, Roose M, Krueger R, Frederici C (2006). Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). Theor. Appl. Genet. 112:1519-1531.

Burt SA (2004). Essential oils: Their antibacterial properties and potential applications in foods: A review. Int. J. Food Microbiol. 94: 223-253.

Chen C, Bowman KD, Choi YA, Dang PM, Rao MN, Huang S, Soneji JR, McCollum TG, Jr FGG (2007). EST-SSR genetic maps for *Citrus sinensis* and *Poncirus trifoliate*. Tree Genet. Genomes 4:1-10.

CLAM (Comité de Liaison de l'Agrumiculture Méditerranéenne) (2007). *Les exportations d'agrumes du basin Méditerranéen. Statistiques, evaluations, repartitions, situation* 2006-2007, 121pp.

CLSI (National Committee for Clinical Laboratory Standards) (2002) Methods for dilution antimicrobial susceptibility tests of bacteria that grow aerobically. Approved Standard M100 -S12. Wayne. PA, NCCLS.

Corazza-Nunes MJ, Machado MA, Nunes WMC, Cristofani M, Targon MLPN (2002) Assessment of genetic variability in grapefruits (*Citrus paradisi*) and pummelos (*C. maxima*) using RAPD and SSR markers. Euphytica 126: 169-176.

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

Dugo G, Di Giacomo A (2002). Citrus: the genus *Citrus*, medicinal and aromatic plants-industrial profiles. Taylor & Francis group, London.

Eid S, Guindy L (2008). Citriculture in Egypt, "safe and high quality supply chains and networks for the citrus industry between Mediterranean partner countries and Europe" http://www2.spi.pt/euromedCitrusnet/Documents/Non_EU_RegionalConferenceProceedings/ANNEX%202.6%20Citriculture%20%20in%20Egypt.pdf

EL-Mouei R, Choumane W, Dway F (2011). Characterization and estimation of genetic diversity in citrus rootstocks. Int. J. Agric. Biol. 13: 571-575.

Fang DQ, Roose ML (1997). Identification of closely related citrus

cultivars with inter-simple sequence repeat markers. Theor. Appl. Genet. 95: 408-417.
FAOSTAT (2012) http://www.fao.org/fileadmin/templates/est/COMM_MARKETS_MONITORING/Citrus/Documents/CITRUS_BULLETIN_2012.pdf
Federici CT, Fang DQ, Scora RW, Roose ML (1998). Phylogenetic relationships within the genus *Citrus* (*Rutaceae*) and related genera as revealed by RFLP and RAPD analysis. Theor. Appl. Genet. 96:812-822.
Froelicher Y, Mouhaya W, Bassene JB, Costantino G, Kamiri M, Luro F, Morillon R, Ollitrault P (2011). New universal mitochondrial PCR markers reveal new information on maternal citrus phylogeny. Tree Genet. Genomes 7:49-61.
Gattuso G, Barreca D, Gargiulli C, Leuzzi U, Caristi C (2007). Flavonoid Composition of Citrus Juices. Molecules 12:1641-1673.
Ghasemi K, Ghasemi Y, Ebrahimzadeh MA (2009). Antioxidant activity, phenol and flavonoid contents of 13 citrus species peels and tissues. Pak. J. Pharm. Sci. 22:277 -281.
Gmitter FG (1995). Origin, evolution and breeding of the grapefruit. Plant Breed. Rev. 13:345-363.
Golein B, Nazeryan M, Babakhani B (2012). Assessing genetic variability in male sterile and low fertile citrus cultivars utilizing simple sequence repeat markers (SSRs). Afr. J. Biotechnol. 11: 1632-1638.
Gulsen O, Roose ML (2001). Lemons: diversity and relationships with selected citrus genotypes as measured with nuclear genome markers. J. Am. Soc. Hortic. Sci. 126:309-317.
Gulsen O, Uzun A, Canan I, Seday U, Canihos E (2010). A new citrus linkage map based on SRAP, SSR, ISSR, POGP, RGA and RAPD markers. Euphytica 173: 265-277.
Hashempour A, Sharifzadeh K, Bakhshi D, Ghazvini RF, Ghasemnezhad M, Mighani H (2013). Variation in total phenolic, ascorbic acid and antioxidant activity of citrus fruit of six species cultivated in north of Iran. Int. J. Agric. Res. Rev. 3: 1-5
Hindi NKK, Chabuck ZAG (2013). Antimicrobial activity of different aqueous lemon extracts. J. Appl. Pharm. Sci. 3:74-78.
Hussein EHA, Abd-alla SMM, Awad NA, Hussein MS (2003). Genetic analysis in some citrus accessions using microsatellites and AFLP-based markers. Arab J. Biotechnol. 6: 203-222.
IPGRI (1999) Descriptors for Citrus. International Plant Genetic Resources Institute, Rome, Italy. ISBN 92-9043-425-2.
Jayana BL, Prasai T, Singh A, Yami KD (2010). Study of antimicrobial activity of lime juice against *Vibrio cholera*. Sci. World 8: 44-46.
Johann S, Oliveira VL, Pizzolatti MG, Schripsema J, Braz Filho R, Branco A, Smânia A (2007). Antimicrobial activity of wax and hexane extracts from *Citrus* spp. peels. Mem. Inst. Oswaldo Cruz. 102:681 - 685.
Kalia RK, Rai MK, Kalia S, Singh R, Dhawan AK (2011). Microsatellite markers: an overview of the recent progress in plants. Euphytica 177:309-334.
Li X, Xie R, Lu Z, Zhou Z (2010). The origin of cultivated citrus as inferred from internal transcribed spacer and chloroplast DNA sequence and amplified fragment length polymorphism fingerprints. J. Am. Soc. Hortic. Sci. 135:341-350.
Nei M (1972) Genetic distance between populations. Am. Nat. 106:283-292.
Nicolosi E, Deng ZN, Gentile ACA, Malfa S, Continella G, Tribulato E (2000). Citrus phylogeny and genetic origin of important species as investigated by molecular markers. Theor. Appl. Genet. 100:1155-66.
Pang XM, Hu CG, Deng XX (2007). Phylogenetic relationships within Citrus and its related genera as inferred from AFLP markers. Genet. Resour. Crop Evol. 54:429-436
Pichaiyongvongdee S, Haruenkit R (2009). Comparative studies of limonin and naringin distribution in different parts of pummelo [*Citrus grandis* (L.) Osbeck] cultivars grown in Thailand. Kasetsart J. Nat. Sci. 43:28-36.
Prescott LM, Harley JP, klein DA (2002). Microbiology 5th Eds, McGraw-Hill New York. pp. 809-811.
Qi XH, Yang JH, Zhang MF (2008). AFLP-based genetic diversity assessment among Chinese vegetable mustards (*Brassica juncea* (L.) Czern.). Genet. Resour. Crop Evol. 55:705-711.
Rekha C, Poornima G, Manasa M, Abhipsa V, Pavithra Devi J, Vijay Kumar HT, Prashith Kekuda TR (2012). Ascorbic acid, total phenol content and antioxidant activity of fresh juices of four ripe and unripe citrus fruits. Chem. Sci. Trans. 1:303-310.
Roose ML (2007). Mapping and marker assisted selection in Citrus. In: Khan IA (ed.) Citrus genetics, breeding and biotechnology. CAB International, Wallingford, UK. pp. 275-286.
Sankar AA, Moore GA (2001). Evaluation of inter-simple sequence repeat analysis for mapping in Citrus and extension of the genetic linkage map. Theor. Appl. Genet. 102:206-214.
Sidana J, Saini V, Dahiya S, Nain P, Bala S (2013). A Review on Citrus - "The Boon of Nature". Int. J. Pharm. Sci. Rev. Res. 18: 20-27.
Siragusa M, Pasquale F, Abbate L, Tusa N (2006). Identification of sour orange accessions and evaluation of their genetic variability by molecular marker analysis. HortScience 41: 84-89.
Sneath PHA, Sokal RB (1973). Numerical taxonomy. The principles and practice of numerical classification. San Francisco: W. H. Freeman.
Snedecor GW, Cochran WG (1980). Statistical Methods. 7th Ed., Iowa State Univ. Press, Amr., USA. pp. 255-269.
Snoussi H, Duval M, Garcia-Lor A, Belfalah Z, Froelicher Y, Risterucci A, Perrier X, Jacquemoud-Collet J, Navarro L, Harrabi M, Ollitrault P (2012). Assessment of the genetic diversity of the Tunisian citrus rootstock germplasm. BMC Genet.13:1-12.
Sultana HS, Ali M, Panda BP (2012). Influence of volatile constituents of fruit peels of Citrus reticulata Blanco on clinically isolated pathogenic microorganisms under *In-vitro*. Asian Pac. J. Trop. Biomed. S1299-S1302
Targon MLPN, Machado MA, Coletta Filho HD, Cristofani M (2000). Genetic polymorphism of sweet orange (*Citrus sinensi* L. Osbeck) varieties evaluated by random amplified polymorphic DNA. Acta Hortic. 535:51-5.
Tomotake H, Koga T, Yamato M, Kassu A, Ota F (2006) Antimicrobial activity of citrus fruits juices against *Vibrio* species. J. Nutr. Sci. Vitaminol. 52:157-160.
Uzun A, Gulsen O, Yesiloglu T, Aka-Kacar Y, Tuzcu O (2010). Distinguishing grapefruit and pummelo accessions using ISSR markers. Czech J. Genet. Plant Breed. 46: 170-177.
Xu G, Liu D, Chen J, Ye X, Ma Y, Shi J (2008). Juice components and antioxidant capacity of citrus varieties cultivated in China. Food Chem. 106:545-551.
Yang Y, Pan Y, Gong X, Fan M (2010). Genetic variation in the endangered *Rutaceae* species *Citrus hongheensis* based on ISSR fingerprinting. Genet. Resour. Crop Evol. 57:1239-1248.

Supplementary Tables

Table S1. Number of accessions per citrus group, total number of amplicons, monomorphic amplicons and polymorphic amplicons as revealed by 13 ISSR primers.

Citrus group	Number of accessions	Total amplicons	Monomorphic amplicons	Polymorphic amplicons
Sweet orange	20	142	63	79
Mandarin	6	164	69	95
Lemon and lime	10	167	48	119
Sour orange	3	114	93	21
Grapefruit	4	126	88	38
Pummelo	5	139	72	67

Table S2. ISSR positive markers that differentiated *Citrus* species.

Primer	*Citrus* species					
	C. sinensis	***Citrus spp.***	***C. limetta***	***C. jambhiri***	***C. aurantifolia***	***C. aurantium***
	ISSR fragment size (bp)					
H_{12}	408, 500, 764, 1305	-	-	-	-	1233
H_{13}	159, 209	-	-	1024, 1302	-	479, 648, 869, 1734
H_{14}	301, 648	-	-	-	-	836
H_{15}	308, 470, 787	477	-	-	-	798
H_{16}	315	-	-	-	-	-
H_{21}	161	-	-	665	1128	416, 443, 537, 754, 912, 1395, 1714, 2115, 2823, 3470
H_{29}	-	-	-	-	1684	646
P_2	1457	1016	536	-	-	661, 812, 1485
P_3	-	-	-	-	-	368, 439, 599, 893
P_4	265, 1248	-	-	-	312, 368	239, 415, 498, 674, 806
P_{11}	198, 292, 913, 1086	798	-	-	-	435, 1174
P_{16}	308, 470, 787	-	-	-	-	798
Total	23	3	1	3	4	33

academicJournals

Vol. 13(26), pp. 2637-2640, 25 June, 2014
DOI: 10.5897/AJB2013.12831
Article Number: C95E74645608
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Short Communication

In vitro development and regeneration of microcorms in saffron (*Crocus sativus* L)

J. I. Mir, N. Ahmed, Wajida Shafi, Rizwan Rashid, Mudasir Hafiz Khan*, M. A. Sheikh, Uzma Noor Shah, Shafiya Zaffar and Irfan Rather

Central Institute of Temperate Horticulture, ICAR, Rangreth, Srinagar-190 007, Jammu & Kashmir, India.

Receive 23 May, 2013; Accepted 25 February, 2014

Saffron (*Crocus sativus* L.) being triploid in nature is propagated by vegetative means through corms. The natural propagation rate of such plant species is relatively low; therefore an *in vitro* multiplication technique like micropropagation has been used as an alternative method of propagation for saffron. In the present investigation, apical bud explants were cultured on different nutrient media supplemented with various concentrations of plant growth regulators. Micro-corm formation was observed on all the media combinations. Maximum number (10) and weight (1.54 g) of microcorms developed were observed on MS media supplemented with 6-benzyl amino purine (BAP) (2 mg/L) + naphthalene acetic acid (NAA) (0.5 mg/L) + paclobutrazol (1.5 mg/L). Cultural conditions under light or in dark did not affect the corm formation and growth. Shoot and root regeneration was observed in the microcorms developed under *in-vitro* conditions. Maximum number of shoot (11.6) and length of shoots (11.4 cm) was also observed on MS media supplemented with NAA (21.6 µM) + BAP (22.2 µM). Maximum number of roots (11) and length of roots (11.4 cm) were obtained on G-5 media containing NAA (21.6 µM) + BAP (22.2 µM). The above observations will be useful as the base to make a possible road way for production of quality planting material in saffron.

Key words: Saffron, growth regulators, micropropagation, apical bud.

INTRODUCTION

Crocus sativus L. (*Iridaceae*) is cultivated in few countries of the world for its highly valued stigmatic lobes. Saffron being triploid (2n = 3x = 24) is sterile and is propagated vegetatively through corms. A corm survives for only one season, producing up to ten "cormlets" that eventually give rise to new plants (Deo, 2003). Corm production is a rate limiting factor in saffron propagation as rate of generation of daughter corms under natural conditions is low (Chahota et al., 2003) which results in limited availability of propagating material for field cultivation. Micropropagation is very good alternative for quality planting material/seed production and large scale multiplication of disease free saffron (Ascough et al., 2009). The successful tissue culture protocol was developed in saffron by several workers (Sharma et al., 2008, Mir et al., 2010). Microcorm production under *in-vitro* conditions shows promise with respect to rate of multiplication and number of microcorms produced in saffron (Darvishi et al., 2007). Regeneration has been described from corm-derived callus cultures via somatic

*Corresponding author. E-mail: drmhkhan8@gmail.com.

Table 1. *In-vitro* corm multiplication in saffron from apical buds.

Medium	BAP mg L^{-1}	NAA mg L^{-1}	Paclobutrazol mg L^{-1}	Number of microcorms	Weight of corm (g)
MS	1.0	0.1	1.0	$8.4^{abc} \pm 0.56$	$0.96^{bc} \pm 0.12$
	1.0	0.5	1.5	$8.4^{abc} \pm 0.25$	$0.6^{cd} \pm 0.35$
	2.0	0.5	1.5	$10.2^{a} \pm 0.40$	$1.54^{a} \pm 0.11$
	2.0	1.0	2.0	$7.6^{bc} \pm 0.25$	$0.48^{cd} \pm 0.02$
G-5	1.0	0.1	1.0	$6.2^{c} \pm 0.48$	$0.72^{cd} \pm 0.10$
	1.0	0.5	1.5	$7.2^{bc} \pm 0.18$	$0.48^{cd} \pm 0.02$
	2.0	0.5	1.5	$8.8^{ab} \pm 0.36$	$1.34^{ab} \pm 0.11$
	2.0	1.0	2.0	$6.0^{c} \pm 0.38$	$0.34^{d} \pm 0.02$

Means followed by the same letter within the columns are not significantly different (P= 0.05) using Duncan's multiple range test.

Figure 1. *In-vitro* corm multiplication in saffron.

embryogenesis, organogenesis and protoplasts (Demeter et al., 2010; Mir et al., 2010) but with low frequencies of normal plant formation. The present study was undertaken for efficient corm multiplication and *in-vitro* regeneration in saffron.

MATERIALS AND METHODS

Explants

The present experiment has been carried out during 2010-2012 at Biotechnology Laboratory of Central Institute of Temperate Horticulture, Rangreth, Srinagar. Saffron corms were grown under controlled conditions in polyhouse of CITH, Srinagar Farm. The bulbs were harvested just before the onset of flowering and apical buds were removed and used for microcorm production and regeneration experiment. Explants were thoroughly washed under running tap water and sterilized by dipping in 70% ethanol for 3-4 min followed by surface sterilization with 0.1% $HgCl_2$ for 10 min and rinsed 5 times with sterile distilled water.

Culture conditions

Basal media employed were (Murashige and Skoog, 1962) and G-5 Gamborg et al., 1968), each at pH 5.8 and with 0.9 or 1% agar. Phytohormones were used at different concentrations. BA (2.22, 22.2, 4.44 and 44.4 µM) and NAA (10.8, 16.2, 21.6 and 27.0 µM) in combinations were used for regeneration and BA (0.5, 1.0, 1.5 and 2.0 mg/L) and NAA (0.1 and 0.5 mg/L) were used for corm multiplication. After preparing the media explants (apical buds) were cultured in glass tubes (90 × 25 mm) and jam bottles (500 ml). Cultures were maintained at 25 ± 1°C under 16/8 h (light/darkness) photoperiod with a light intensity of approximately 4000 lux.

Microcorm formation and regeneration

Cultures were sub-cultured and transferred to fresh media after every 4 weeks. Observations with respect to shoot length, number of shoots, root length, number of roots, number of corms and weight of corms were taken after every six weeks. This study was carried out as a factorial experiment based on completely randomized design (CRD) with 48 treatments in 5 replications.

Statistical analysis

Each treatment was replicated 5 times and observations in stages of development were recorded periodically. The data was analyzed by comparing means using one way analysis of variance (ANOVA) and the significance was determined by Duncan's Multiple Range Test using SAS (v 9.2).

RESULTS AND DISCUSSION

Corm multiplication

Maximum number (10) and weight (1.54 g) of microcorms developed were observed on MS media supplemented with 2 mg/L BAP + 0.5 mg/L NAA + 1.5 mg/L paclobutrazol followed by 8.8 average number of microcorms with 1.34 g average weight on G-5 media supplemented with 2 mg/L BAP + 0.5 mg/L NAA + 1.5 mg/L paclobutrazol (Table 1, Figure 1). Cultural conditions under light or in dark did not affect the corm formation and growth. Our results showed further

Table 2. *In-vitro* shoot and root regeneration in saffron from apical buds.

Medium	NAA (µM)	BA (µM)	Shoot length (cm)	Number of shoots	Root length (cm)	Number of roots
MS	27	44.4	$10.8^{ab} \pm 0.53$	$10^{ab} \pm 0.57$	$7.6^{c} \pm 0.43$	$7.4^{bc} \pm 0.53$
	21.6	22.2	$11.4^{a} \pm 0.51$	$11.6^{a} \pm 0.64$	$10^{ab} \pm 0.38$	$9.8^{ab} \pm 0.43$
	16.2	4.4	$10.0^{abc} \pm 0.35$	$8.8^{abc} \pm 0.69$	$8.4^{bc} \pm 0.20$	$7^{bc} \pm 0.35$
	10.8	2.22	$6.8^{de} \pm 0.43$	$6.6^{bc} \pm 0.56$	$7.2^{c} \pm 0.18$	$5.8^{c} \pm 0.36$
G-5	27	44.4	$8.4^{bcd} \pm 0.25$	$8^{abc} \pm 0.57$	$10.2^{ab} \pm 0.43$	$11.0^{a} \pm 0.89$
	21.6	22.2	$10.4^{abc} \pm 0.37$	$10^{ab} \pm 0.52$	$11.40^{a} \pm 0.46$	$11.4^{a} \pm 0.53$
	16.2	4.4	$08^{cde} \pm 0.35$	$6.8^{bc} \pm 0.55$	$10.0^{ab} \pm 0.22$	$8.8^{abc} \pm 0.53$
	10.8	2.22	$5.6^{e} \pm 0.37$	$5.4^{c} \pm 0.49$	$8.8^{bc} \pm 0.18$	$7.4^{bc} \pm 0.37$

Means followed by the same letter within the columns are not significantly different (P= 0.05) using Duncan's multiple range test.

Figure 2. *In-vitro* shoot (a & b) and root (c & d) regeneration.

improvement over the protocols developed earlier (Mir et al., 2010). *In vitro* micro-corm production of saffron has been obtained by culturing leaf segments and apical buds (Sharma et al., 2008), shoot explants (Milyaeva et al., 1995) and ovary explants (Raja et al., 2007). Microcorm formation from apical bud takes only eight months under *in-vitro* conditions as against 22 months under field conditions.

Shoot and root regeneration

Apical buds started sprouting within 10 days of incubation on culture media; however, only those cultured at appropriate concentrations of NAA and BA produce multiple shoots. Maximum number of shoot (11.6) and length of shoots (11.4 cm) was also observed on MS media supplemented with 21.6 µM NAA + 22.2 µM BAP (Table 2, Figure 2). In the present investigation, the results on shoot regeneration revealed that NAA and BA are essential for shoot regeneration of saffron (*Crocus sativus* L.).

The apical buds inducing multiple shoots (11.6) with length of 11.4 cm was obtained in our studies were higher than that reported earlier from corms (Chauhan et al., 1999), isolated buds (Ascough et al., 2009), ovaries (Demeter et al., 2010) or apical buds (Sharma et al., 2008).

Auxins in combination with cytokinins can greatly influence the frequency of regeneration (Raja et al., 2007; Abbas and Qaiser, 2012; Sivanesan and Jeong, 2012).

Majourhay et al. (2007) investigated the ability of different cytokinins to induce shoot formation. Shoot development on corm explants was promoted by cytokinins while corm formation and growth was promoted by ethylene exposure (Plessner et al., 1990). Maximum number of roots (11) and length of roots (11.4 cm) were obtained on G-5 media containing NAA (21.6 µM) + BAP (22.2 µM). Our results on root multiplication and elongation are better than that reported earlier from corms (Sharma et al., 2008; Raja et al., 2007). The above observations will be useful as the base to make a possible road way for production of quality planting material in saffron. *In-vitro* regeneration in saffron was reported earlier by Mir et al. (2010); Devi et al. (2011); Ahouran et al. (2012); Cavusoglu et al. (2013); Sivanesan et al. (2014).

Our results on micro-corm production and multiplication promise to bridge the gap between land availability for saffron cultivation and availability of quality saffron planting material and regeneration protocol, with help in the development and rapid clonal propagation of novel saffron plant material.

Conflict of Interests

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

REFERENCES

Abbas H, Qaiser M (2012). *In vitro* response of *Ruellia bracteolata* to different growth hormones - an attempt to conserve an endangered species. Plant Cell Tiss. Organ Culture 44(2):791-794.

Ahouran M, Hosseini R, Zarghami R (2012). Corms as a source of explants for the successful clonal propagation of Crocus cancellatus. J. Crop Sci. Biotechnol. 15(1):47-51.

Ascough GD, Erwin JE, Staden J (2009). Micropropagation of *Iridaceae*-a review. Plant Cell Tissue and Organ Culture 97:1-19.

Cavusoglu A, Sulusoglu M, Erkal S (2013). Plant regeneration and corm formation of saffron (*Crocus sativus* L.) *in vitro.* Res. J. Biotechnol. 8(12):128-133.

Chahota RK, Dhiman KC, Rana SS, Singh M (2003). Efficacy of different propagating methods for higher daughter corm production in saffron (*Crocus sativus* L.). Indian Perfumer 47:155-158.

Chauhan RS, Sharma TR, Chahota RK, Singh BM (1999). *In vitro* cormlet production from micro-propagated shoots of saffron (*Crocus sativus* L.). Indian Perfumer 43:150-155.

Darvishi E, Zarghami R, Mishani CA, Omidi M (2007). Effects of different hormone treatments on non-embryogenic and embryogenic callus induction and time-term enzyme treatments on number and viability of isolated protoplasts in saffron. (*Crocus sativus* L.). Acta Horti. 739:279-284.

Demeter Z, Gyula Suranyi V, Molnar A, Sramko G, Beyer D, Konya Z (2010). Somatic embryogenesis and regeneration from shoot primordia of *Crocus heuffelianus*. Plant Cell Tiss. Organ Culture 100:349-353.

Deo B (2003). Growing Saffron – The World's Most Expensive Spice, Crop & Food Research (New Zealand Institute for Crop & Food Research), no. 20, http://www.crop.cri.nz/home/productsservices/publications/broadsheets/020 Saffron.pdf.

Devi K, Sharma M, Sing M, Ahuja PS (2011). *In vitro* cormlet production and growth evaluation under greenhouse conditions in saffron (*Crocus sativus* L.) -A commercially important crop. Eng. Life Sci. 11:189-194.

Gamborg O, Miller R, Ojima K (1968). Nutrient requirement suspensions cultures of soybean root cells. Exp. Cell Res. 50:151-158.

Majourhay K, Fernandez JA, Martınez-Gomez P, Piqueras A (2007). Enhanced plantlet regeneration from cultured meristems in sprouting buds of saffron corms. Acta Horti. 739:275-278.

Milyaeva EL, Azizbekova NS, Komarova EN, Akhundova DD (1995). *In vitro* formation of regenerant corms of Saffron (*Crocus sativus* L.). Russian Journal of Pl. Physio. 42:112-119.

Mir JI, Ahmed N, Rashid R, Wani SH, Itoo H, Sheikh MA (2010). Corm multiplication and development of stigma like structures under *In vitro* conditions in saffron. In*:* Proceedings of National Symposium on Botanical Researches- Present Scenario. Department of Botany, Punjabi University, Patiala. 143:18-19

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15:473-497.

Plessner O, Ziv M, Negbi M (1990). *In vitro* corm production in the saffron crocus (*Crocus sativus* L.). Plant Cell Tiss. Organ Culture 20:89-94.

Raja W, Zaffer G, Wani SA (2007). *In vitro* microcorm formation in saffron (*Crocus sativus* L.) Acta Horti. 739:291-296

Sharma KD, Rathour R, Sharma R, Goel S, Sharma TR, Singh BM (2008). *In vitro* cormlet development in *Crocus sativus*. Biologia Plantarum 52:709-712.

Sivanesan I, Jana S, Jeong BR (2014). *In-vitro* shoot regeneration and microcorm development in *Crocus vernus* (L.) hill. Pak. J. Bot. 46(2):693-697.

Sivanesan I, Jeong BR (2012). Identification of somaclonal variants in proliferating shoot cultures of *Senecio cruentus*cv. Tokyo Daruma. Plant Cell Tiss. Organ Culture 111:247-253.

academicJournals

Vol. 13(26), pp. 2582-2592, 25 June, 2014
DOI: 10.5897/AJB2013.13003
Article Number: BFE87F145603
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Review

Microbes as interesting source of novel insecticides: A review

B. Ratna Kumari, R. Vijayabharathi, V. Srinivas and S. Gopalakrishnan*

Grain Legumes, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324, Andhra Pradesh, India.

Received 5 July, 2013; Accepted 10 June, 2014

Microbes are ubiquitous, survive in all sorts of environments and have a profound influence on the earth. In the present day plant protection scenario, development of resistance to chemical pesticides is the major hurdle in insect pest management. In recent years, several microbes with potential insecticidal properties have come to light. Viruses, bacteria, fungi and protozoa that are known to produce an array of metabolites or toxins, form the basis for microbial insecticides. Since these versatile organisms are amenable for genetic engineering, strains with good insecticidal properties can be identified, evaluated and utilized for pest control. This paper reviews the insecticidal properties of microbes and their potential utility in pest management.

Key words: Microbes, insecticides, metabolites, pest management.

INTRODUCTION

Plant pests and diseases have a serious effect on food production-global crop yields are reduced by 20 to 40% annually due to plant pests and diseases (FAO, 2012). Indiscriminate use of pesticides to combat pest challenges have increased the selection pressure leading to resistance in insects. In such a situation, alternate options of pest control are much awaited. Though biopesticides cover about 1% of the total plant protection products globally, their number and the growth rate have been showing an increasing trend in the past two decades (Ranga Rao et al., 2007). About 175 biopesticide active ingredients and 700 products have been registered worldwide. Among various bio-products, *Bacillus thuringiensis* (Bt), *Trichoderma viride*, *Metarhizium* spp., *Beauveria bassiana* and nuclear polyhedrosis virus are popularly used in plant protection (Anonymous, 2007). Microbes often produce epizootics which is a natural control phenomenon of some insect pests. The need of the hour is development of environment-friendly, microbe-based insecticides, which act differently from known chemicals, thereby providing insect the least chance to develop resistance. So far, prevailing microbial pesticides are being used mainly as foliar applicants. However, new products suitable for varied methods of application such as seed treatment, whorl application, bait treatment etc. are essential. Microbial-based pesticides, their mode of action, application in pest control are described below.

*Corresponding author. E-mail: s.gopalakrishnan@cgiar.org. Tel: +91 9177406003.

VIRUSES

Baculoviruses, which are considered to be the largest and most broadly studied insect viruses, are environment-friendly insect control agents. Individual isolates normally show a limited host range and infect only closely related species (Chang et al., 2003). They induce lethal epizootics that can devastate host insect populations and were used successfully to control different insect pests. Nucleopolyhedrovirus and Granulovirus were isolated from lepidopteran insects. The viral insecticide Elcar™ (*Heliothis zea* NPV) introduced during 1980s, provided control of cotton bollworm; its production and usage was later limited. Another baculovirus, HaSNPV (*Helicoverpa armigera* single nuclear polyhedrosis virus), almost identical to HzSNPV (*H. zea* single nuclear polyhedrosis virus), was registered in China as a pesticide in 1993 (Zhang et al., 1995) and has been extensively used on cotton and many crops in India (Grzywacz et al., 2005; Rabindra et al., 2005; Srinivasa et al., 2008). Commercial preparations based on *Spodoptera* NPV were used to protect cotton, corn and vegetables globally (Moscardi, 1999; Kumari and Singh, 2009). *Autographa californica* and *Anagrapha falcifera* NPVs with relatively broad host spectrum activity were used on a variety of crops infested with *Spodoptera* and *Helicoverpa*.

Granulovirus CpGV was effective against the coddling moth (*Cydia pomonella*) on fruit crops. Another granulovirus (GV), *Erinnyis ello* (cassava hornworm) granulovirus, was found to be very efficient for protection of cassava plantations (Bellotti, 1999) in South American countries. The GVs of tea tortricids, *Homona magnanima* and *Adoxophyes honmai* were used in Japan. Entomopoxvirus, a large DNA genome virus is found pathogenic to insects. *Amsacta moorei* entomopoxvirus, has been reported to infect agriculturally important lepidopteran pests such as *Estigmena acrea* and *Lymantria dispar* (Muratoglu et al., 2010). Among all the viruses studied, NPV was effective on lepidopteran pests in different agroecosystems. However, favourable weather, slow action and microbial contamination are the major constraints in baculovirus production and use. To address such constraints, engineering viruses to express insecticidal genes is one of the best approaches.

Recombinant viruses

The insertion or deletion of a single gene from the viral genome can alter the biological properties of the virus. Insertion of insect-specific toxin genes such as juvenile hormone esterase, diuretic hormone and prothoracicotropic hormone, genes encoding enzyme inhibitors, neuropeptides or toxins improve the efficiency of viruses. *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV) recombinants with wild type and mutated mutated versions of Juvenile Hormone Esterase (JHE) reduced the consumption of food by *H. virescens* and *Trichoplusia ni* (Bonning et al., 1999). The gene coding for a toxin from scorpion *Androctonus australis* reduced the feeding damage by about 40-60% in lepidopteran larvae (Choi et al., 2008). Toxin genes isolated from other scorpions, for example *Leiurus quinquestriatus hebraeus* (Imai et al., 2000), straw itch mite *Pyemotes tritici* (Burden et al., 2000), ants (Szolajska et al., 2004) or spiders (Prikhod'ko Prikhod'ko et al., 1998), sea anemones and *B. thuringiensis* (Chang et al., 2003) have been intensively studied as potential enhancers of baculovirus activity. Genetically modified *Anticarsia gemmatalis* multicapsid nuclear polyhedrosis virus (AgMNPV) by the inactivation of ecdysteroid glucosyltransferase (egt) gene, were shown to kill infected larvae more rapidly when compared to wild-type virus infections (Pinedo et al., 2003). Ignoffo et al. (1995) found that AcMNPV *pp34* deletion mutants have an increased infectivity in *Trichoplusia ni* larvae due to the increased loads of occlusion-derived virions (ODV) from the polyhedra in the midgut to start the infection process. Application of recombinant baculoviruses, vAPcmIT2 and vAP10IT2 against two major pesticide-resistant vegetable pests, *Plutella xylostella* (Linnaeus) and *S. exigua* resulted in shortening of the lethal time (Tuan et al., 2007).

Two recombinant baculoviruses containing the ScathL gene from *Sarcophaga peregrina* (vSynScathL) and the keratinase gene from the fungus *Aspergillus fumigatus* (vSynKerat), against third-instar and neonate *S. frugiperda* larvae showed protease activity in the haemolymph and reduced the time of kill (Gramkow et al., 2010). Report of Seo et al. (2005) documented higher pathogenicity for recombinant baculovirus containing a fusion protein with polyhedrin and Bt toxin than wild type strains.

BACTERIA

Bacillus thuringiensis

Among the bacteria, *B. thuringiensis* (Bt) is the most important and also the most extensively studied and used in the integrated pest management programs. It has long been regarded as a bonafide entomopathogen that can produce an array of virulence factors including insecticidal parasporal crystal (Cry) toxins, vegetative insecticidal proteins, phospholipases, immune inhibitors and antibiotics. Bt produces delta-endotoxin that reacts with the cells of the gut lining of susceptible insects. There are about 200 registered Bt products in the USA and, at the end of the last century, worldwide sales amounted to about 100 million dollars (about 2% of the total global insecticide market) (Anonymous, 1998). Though this versatile organism has multiple insecticidal

properties, Ultra violet (UV) radiation inhibits its activity. UV resistant mutant strains with high melanin, which absorb light of any wavelength, can be used for large scale production of light stable insecticides (Liu et al., 2013). Other than Cry toxins, orally active insecticidal proteins that meet the efficacy hurdles required for pest control have also been reported from Bt. Vegetative insecticidal proteins (Vips) are produced by *B. cereus* and *B. thuringiensis* which show similar activity to endotoxins. Vip1and Vip2 are toxic to coleopteran insects and Vip3 is toxic to lepidopteran insects (Zhu et al., 2006). VIPs have excellent activity against black cutworms and armyworms (Yu et al., 1997) *S. frugiperda* (Barreto et al., 1999). *S. litura* and *Plutella xylostella* (Bhalla et al., 2005), *Helithios zea, Trichoplusia ni*, *Ostrinia nubilalis* (Fang et al., 2007; Sellami et al., 2011).

Insecticidal bacteria from nematodes

Another interesting source of microbial insecticides is bacterial symbionts of nematodes. *Xenorhabdus* and *Photorhabdus* are gram-negative bacteria that live in nematodes are highly toxic to many insect species (Burnell and Stock, 2000). The bacteria and the nematodes produce a variety of metabolites to enable them to colonize and reproduce in the insect host. The metabolites produced include enzymes such as proteases, lipases and phospholipases to maintain a food supply during reproduction (Bowen et al., 2000), and antifungal and antibacterial agents to prevent degradation or colonization of the insect carcass while the bacteria and nematodes reproduce. The bacteria such as *B. thuringiensis* produce Bt and Vip toxins against insects (Chattopadhyay et al., 2004); similarly, nematodes also produce toxins called Toxin Complex (Tc) by their symbiotic bacterial partners *X. nematophilus* and *P. luminescens* (French-Constant and Bowen, 2000).

Bowen (1995) reported that a soluble protein fraction derived from *P. luminescens* culture medium possessed sufficient insecticidal activity to kill *Manduca sexta* upon injection. The bacterium *Xenorhabdus nematophila* produces novel secreted protein toxin which was found effective against *Galleria mellonella* and *H. armigera*, cabbage white caterpillar *Pieris brassicae*, mosquito larva *Aedes aegypti* and mustard beetle *Phaedon cochleariae* (Sergeant et al., 2006). The reports show that these bacteria are effective on most of the economically important lepidopteran, dipteran and coleopteran insect orders. Hence, there is a wide scope to harness these organisms in the insect pest management. The genome of *P. luminescens* was homologous to hemolysin A, chitinase, Rtx (repeats-in-toxin)-like toxin, and delta endotoxin (Duchaud et al., 2003). It is interesting that novel colicin and immunity proteins are also found associated with insecticidal Tc genes in *Photorhabdus* spp. (Sharma et al., 2002). In future, in areas where the pests are resistant to Bt, these bacteria from nematodes could be the successful option to replace Bt.

Bacteria supplementing the activity of other bacteria

In some instances, one microbe assists other microbe to elevate its significance. In the absence of indigenous midgut bacteria, *B. thuringiensis* does not kill larvae. Elimination of the gut microbial community by oral administration of antibiotics abolished *B. thuringiensis* insecticidal activity, and reestablishment of the same gut bacteria that normally resides in the midgut microbial community restored *B. thuringiensis*-mediated killing (Broderick et al., 2006). Thus, the gut microflora plays an important role in the insecticidal properties of Bt. This is an area where the major research is being focused these days (Broderick et al., 2009; Patil et al., 2013).

Insecticidal bacterial flora of insects

Isolation of insecticidal bacterial flora from the insect itself and its use against the same is becoming popular. Several pathogenic bacteria species from insect samples have been developed as pesticides and used successfully in biological control of insects worldwide (Thiery and Frachon, 1997). A common soil organism, *B. cereus*, has been found pathogenic to insects on several occasions, and this species has been isolated from several insect species (Kuzina et al., 2001; Sezen et al., 2005). The isolates *B. cereus* (Ags1), *Bacillus* spp. (Ags2), *B. megaterium* (Ags3), *Enterobacter aerogenes* (Ags4), *Acinetobacter calcoaceticus* (Ags5), *Enterobacter* spp. (Ags6), *Pseudomonas putida* (Ags7), *Enterococcus gallinarum* (Ags8) and *Stenotrophomonas maltophilia* (Ags9) identified from the flora of *Agrotis segetum* when applied on the same, found to cause 60% mortality after eight days of application (Sevim et al., 2010).

Likewise, *B. megaterium* is also common in insect populations (Osborn et al., 2002). Several species of *Enterobacter* have been isolated from several insect species and used for biological control purposes (Sandra and Douglas, 2004; Bahar and Demirbag, 2007). *B. cereus*, *B. sphaericus*, *Morganella morganii*, *Serratia marcescens* and *Klebsiella* species isolated from the crop of predatory larvae of the antlion species *Myrmeleon bore* (Neuroptera: Myrmeleontidae) killed 80% or more cutworms *S. litura* (Nishiwaki et al., 2007). The bacterial flora *Leclercia adecarboxylata* of Colorado potato beetle showed highest insecticidal effect (100% mortality) within five days (Muratoglu et al., 2009) and has a potential for the control of several coleopteran pests. *P. pseudoalcaligenes* strain PPA (first isolated from yellow-spined bamboo locust, *Ceracris kiangsu*, in 1991) is an effective biological controlagent for locust (Zhang et al., 2009). Such isolates can rather be multiplied on large scale and used as foliar applications in field to manage

important insect pests.

Other bacteria with insecticidal properties

Studies have shown mosquitoes to be the major targets of *Lysinibacillus sphaericus (B. sphaericus)* bacterium that produce insecticidal toxins during the vegetative phase of growth. Sphaericolysin, a toxin from the *L. sphaericus* was found lethal to the common cutworm *S. litura* (Nishiwaki et al., 2007). In addition to endotoxins showing insecticidal properties in Bt, there are bacteria which produce insecticidal exotoxins. The exotoxins of microbial origin from *Pseudomonas* spp. toxic to larvae of mosquitoes as well as lepidopteran insects (Murty et al., 1994) were known to act on the haemolymph proteins. *P. aeruginosa* oxyR mutant revealed its ability to kill the insect *Drosophila melanogaster* (Lau et al., 2003). *P. aeruginosa* strain confers an efficient protection against *Galleria mellonella* and *Batocera oleae* (George et al., 2000; Mostakim et al., 2012) and the potency was due to the presence of quantitatively as well as qualitatively different proportions of bio-surfactants in the crude glycolipids (Desai and Banat, 1997). *B. subtilis*, *B. amylofaciens*, *B. megaterium* and *Pseudomonas* spp. were reported to show more than 50% mortality in *S. litura* and *H. armigera* (Gopalakrishnan et al., 2011). Chitinase is one of the virulent factors in entomopathogens and it is positively correlated with insecticidal activity. Research focused on identifying isolates producing chitinase adds to the benefit of recognition of strains with high insecticidal activity. For example, Bahar et al. (2011) identified chitinase-positive bacteria such as *Serratia marcescens* to be active in killing the coleopteran insects with more chitin in their exoskeleton.

Actinomycetes

Actinomycetes form a large group of Gram-positive bacteria that grow as hyphae like fungi. They play an important role in the biological control of insects through the production of insecticidal compounds. The mortality of insect by actinomycetes may be due to secretion of bioactive materials which stimulate the gamma amino butyric acid (GABA) system or disruption of nicotinic acetylcholine receptors (Herbert, 2010). Actinomycetes was found effective against the house fly *Musca domestica* (Hussain et al., 2002), mosquito larvae (Sundarapandian et al., 2002; Dhanasekaran et al., 2010), and *Drosophila melanogaster* (Gadelhak et al., 2005).

Many actinomycetes strains caused larval mortality of the cotton leaf worm *S. littoralis* (Bream et al., 2001). In addition, considerable lethal effect of some actinomycetes was observed on pupae. Actinomycetes are very well known and successfully exploited as a source of secondary metabolites. The secondary metabolites strain of Streptomyces inhibited the growth of *S. exigua, Dendrolimus punctatus, Plutella xylostella, Aphis glycines* and *Culex pipiens* (Huamei et al., 2008). Besides exhibiting insecticidal activity, Streptomyces metabolites also play the role as antimicrobial compounds for insects and hence protect them from microbial pathogens. For instance, beewalf wasps has been found with antibiotic-producing *Streptomyces philanthi* within specialized glands on its antenna and the antibiotics excreted by *S. philanthi,* into the cocoons, protects the beewalf larvae from harmful pathogens (Kroiss et al., 2010). The potential of using chitinase-producing non-Streptomycete actinomycetes belonging to the genus Actinoplanes, which have chitin as a major component of their cuticle, has been investigated for the biological control of insects in the Arabian Gulf area (Gadelhak et al., 2005).

The insecticidal activity of tetranectin, avermectins, faerifungin and macrotetrolides isolated from *Streptomyces aureus*, *S. avermitilis*, *S. albidum* and *S. griseus* respectively, have been reported. Spinosad is a novel insecticide produced from a family of natural products derived from fermentation of the actinomycetes *Saccharopolyspora spinosa* (Snyder et al., 2007) has been accepted in organic farming. It is a neurotoxin with a novel mode of action involving the nicotinic acetylcholine receptor and probably GABA receptors as well (Salgado, 1998).

Exposure causes a cessation of feeding, some 24 hours later, by paralysis and death. It is primarily a stomach poison with some contact activity and is particularly toxic to Lepidoptera and Diptera. The avermectins are a series 16-membered macrocyclic lactone derivatives with potent anthelmintic and insecticidal properties generated as fermentation products by *S. avermitilis* (Pitterna et al., 2009). Cholesterol oxidase derived from Streptomyces broth has shown to have selective, high potency against cotton boll weevil, stunting effect in *H. virescens, H. zea* and *Pectinophora gossypiella* which might be due to disruption of the midgut epithelial membrane (Purcell et al., 1993).

FUNGI

Fungi affect all groups of insects and over 700 species have been recorded as insect pathogens. Fungi do not have to be ingested to infect their host but invade directly through the cuticle, and so can, potentially, be used for the control of all insects including sucking insects. The first step is invasion of host through cuticle by mechanical pressure and enzymatic degradation. Most entomopathogenic fungi are best used when total eradication of a pest is not required, but instead insect populations are controlled below an economic threshold, with some crop damage being acceptable. In addition entomopathogenic fungi have an essential role in Integrated Pest Management (IPM) if they can be used in conjunction

with other strategies for sustainable pest control. A substantial number of mycoinsecticides and mycoacaricides have been developed worldwide since the 1960s. Products based on *B. bassiana* (Li et al., 2001), *M. anisopliae*, *Isaria fumosorosea* and *B. brongniartii* are the most common among the 171 products (Faria and Wraight, 2001) available in the market. Strains of the species *B. bassiana*, *M. anisopliae*, *Verticillium lecani, Nomuraea releyi* and *Paecilomyces fumosoroseus* are currently used for insect control (Lacey and Neven, 2006).

Many of the genera of entomopathogenic fungi either belong to the class Entomophthorales in the Zygomycota or the class Hyphomycetes in the Deuteromycota. Most of the entomopathogenic fungi have life cycles which synchronise with insect host stages and environmental conditions. Some of these fungi are obligate; for example, *Aschersonia aleyrodes* infects only scale insects and whiteflies, while other fungal species are facultative with individual isolates being more specific to target pests. Hence, different preservation methods must be standardized to maintain the viability of the most potent isolates. Sub culturing is the best method of conservation of pathogenicity of fungi. For long term storage, glycerol freeze was proved to be good (Oliveira et al., 2011).

Entomopathogenic Hyphomycete fungi, such as *B. bassiana*, are naturally occurring in soil (Klingen et al., 1998) and the fungus is being developed as a biocontrol agent against soil dwelling pests such as scarabs and weevils (Keller, 2000) with no effect on the non-targeted insects (Goettel and Hajek, 2001). Hosts of agricultural and forest significance include the Colorado potato beetle, the codling moth, several genera of termites, American bollworm *H. armigera* (Thakur and Sandhu, 2010), *Hyblaeapara* and *Eutectona machaeralis*, *Ostrinia nubilalis*, pine caterpillars *Dendrolimus* spp. and green leafhoppers *Nephotettix* spp. Spores of entomopathogenic fungi are available as an emulsified suspension or wettable powder and they are applied via spraying. These fungal spores parasitize into a wide range of insects and pests and hence considered as nonselective biological insecticide. *Lecanicillium* (*Verticillium) lecanii* and *Isaria* (*Paecilomyces) fumosoroseus* fungi mainly attacks sucking pests such as aphids and whiteflies (Kim et al., 2002; Nunez et al., 2008) *Isaria* (*Paecilomyces) fumosoroseus* has strong epizootic potential against *Bemisia* and *Trialeurodes* spp. in both greenhouse and open field environments (Faria and Wraight, 2001). Entomopathogenic fungus *P. tenuipes* was documented to control chemical resistant whiteflies on greenhouse vegetables.

Metarhizium spp. popularly known as green muscardine fungus is known to have potential to control several economically important insect pests of global importance viz., *H. armigera*, *S. litura*, that attack crops such as groundnut, soyabean, sunflower, cotton and tomato (Sahayaraj and Borgio, 2010; Revathi et al., 2011). A complete bioactivity of *M. anisopliae* has been tested on teak skeletonizer, *Eutectona machaeralis,* and found to be a potential myco-biocontrol agent of teak pest (Sandhu et al., 2000).

Nomuraea rileyi can cause epizootic death in various insects. It has been shown that many insect species belonging to Lepidoptera including *S. litura* and some belonging to Coleoptera are susceptible to *Nomuraea rileyi* (Ignoffo, 1981). Its mode of infection and development have been reported for several insect hosts such as *Trichoplusia ni*, *H. zea*, *Plathypena scabra*, *Bombyx mori*, *Pseudoplusia includens, Anticarsia gemmatalis, Spilosoma* (Mathew et al., 1998) and hedge plant eater *Junonia orithya* (Rajak et al., 1991). Information on entomopathogenic fungi and their insect hosts is furnished in Table 1.

Fungal metabolites

Fungi also produce secondary metabolites, an inherent property of the organism and these metabolites exhibit insecticidal activities (Vey et al., 2001). For entomopathogens producing these toxins, infection has been shown to result in more rapid host death compared to strains that do not produce these metabolites (Kershaw et al., 1999). The information on toxins or secondary metabolites produced by fungi is furnished in Table 2.

Entomopathogenic fungi (EPF) produce enzymes for converting insect tissue into nutrients for their growth. Catalyzing activity using specific enzymes is considered one of the main mechanisms of fungal infection to insect host. There is large scope for isolates which could produce extracellular enzymes to degrade the host cuticle in pest management. For instance, *M. anisopliae* grown in optimum fermentation conditions could produce host degrading enzymes such as acid phosphatase and phosphatase isoenzymes (Li et al., 2007; Strasser et al., 2000).

Trichoderma produces protease (31 kDa) and chitinase (44 kDa) during the growth phase (Shakeri and Foster, 2007) and it is also known to produce a number of antibiotics, such as trichodermin, trichodermol, harzianum A, harzianolide and peptaibols (Hoell et al., 2005) which were insecticidal on *Tenebrio molitor*. Tanned insect cuticle is poorly utilizable by most fungi; but the EPF which invade the insect host through its cuticle, have evolved powerful cuticle degrading enzymes such as chymoelastase. The crude *Alternaria alternata* chitinase showed 82% mortality against fruitfly (Sharaf, 2005). Quesada-Moraga et al., (2006) used the crude protein extracts of *M. anisopliae* for the control of *S. litura* and Hu et al., (2007) proved contact toxicity to *S. litura. Tolypocladium* and *Isaria fumosorosea* have proved to be toxic to *Plutella xylostella* (Bandani and Butt, 1999; Freed et al., 2012).

Table 1. Entomopathogenic fungi and their insect hosts.

Fungus	Insect	Reference
Beauveria bassiana	Red flour beetle (*Triboleum castaneum*)	Akbar et al., 2005
B. brongniartii, B. bassiana	*Ceratitis capitata*	Konstantopoulou and Mazomenos, 2005
Nomuraea rileyi, Mucor hiemalis and *Penicillium chrysogenum*	*H. armigera, Ceratitis capitata* and *Bactrocera oleae*	Vimala Devi, 2001
B. bassiana and *Clonostachys rosea*	Coffee berry borer	Vega et al., 2008
Verticillium lecanii	*Macrosiphum euphorbiae*	Askary et al., 1998
Lecanicillium muscarium	*M. euphorbiae* and *Aphidius nigripes*	Askary and Yarmand, 2007
L. longisporum	*Myzus persicae* and *Aphis gossypii*	Kim et al., 2007, 2008
L. attenuatum	*Macrosiphum euphorbiae*	
Lecanicillium spp. DAOM 198499	*Aulacorthum solani*	
L. lecanii	*Coccus viridis*	Vandermeer et al., 2009
Aspergillus flavus	*Culex quinquefasciatus*	Govindarajan et al., 2005
A. niger	*Anopheles aegypti, Culex quinquefasciatus*	Seleena and Lee, 1994
Chrysosporium tropicum	*Anopheles stephensi*	Priyanka et al., 2001

Table 2. Secondary metabolites of fungi effective against insects.

Organism	Metabolites	Insects controlled	Reference
Beauveria spp.	Bassianin, beauvericin, bassianolide, beauveriolide, bassiacridin, oosporein, and tenellin	*Culex pipiens, Aedes aegypti, Calliphora erythrocephala, H. zea*	Quesada-Moraga and Vey, 2004
Paecilomyces fumosoroseus	Pecilomicine-B	*Trialeurodes vaporariorum*	Yankouskaya, 2009
Hirsutella thompsonii	Hirsutellin A, hirsutellin B, phomalatone	Mites	Mazet et al., 1995
Aschersonia aleyrodis and *A. tubulata*	Destruxins, dustanin and homodestruxins.	Whitefly	Boonphong et al., 2001
Trichoderma	Trichodermin, trichodermol, harzianum A, harzianolide, and peptaibols	*Tenebrio molitor*	Shakeri and Foster, 2007

Biofumigants

Some fungi produce volatile insecticidal compounds. This property prompts their use as fumigants for stored pest control. The use of *Muscodor albus* as a biofumigant agent for the control of storage insects has been proved (Lacey and Neven, 2006). The fungus produces a mixture of antimicrobial volatile organic chemicals and when tested against potato tuber moth with 15 or 30 g of *M. albus*, development to the pupal stage was reduced by 61.8 and 72.8%, respectively, relative to controls. Three species of *Muscodor* and one *Gliocladium* sp. that produce volatile organic compounds with biocidal activity have been isolated from several host plants in geographically diverse areas (Daisy et al., 2002; Stinson et al., 2003). Daisy et al. (2002) also showed that naphthalene, an insect repellent, is produced by a related fungus, *Muscodor vitigenus*.

Endophytes

The occurrence of endophytic microbes with pesticidal abilities is leading to exciting new opportunities because it overcomes delivery issues often associated with biopesticides. Endophytic biocontrol agents can be cheaply introduced into seeds, tissue culture plantlets and other propagating material, providing some protection for the microbe to the adversities of the external abiotic and biotic environment. Endophytes can also have additional beneficial properties, such as accelerating seedling emergence, promoting plant growth and tolerance to adverse conditions (Harman, 2011; Companta et al., 2010). Fungal endophytes are quite common in nature and several roles have been recognised, including providing protection against herbivorous insects, plant parasitic nematodes (Elmi et al., 2000), and plant pathogens (Dingle and McGee, 2003; Wicklow et al., 2005). It has been shown that endophytic *B. bassiana* is compatible with both Bt and carbofuran applications used to suppress *Ostrinia nubilalis* (Lewis et al., 1996). Use of Bt transgenic corn did not have any detectable effect on the establishment of *B. bassiana* as a corn endophyte (Lewis et al., 2001). Endophytic *B. bassiana* caused no mortality to

Table 3. Fungal endophytes active against insects.

Fungal endophyte	Insect	References
Neotyphodium	*Rhopalosiphum padi* and *Metopopophium dirhodum*	Clement et al., 2005
Ryegrass endophytes, *Neotyphodium* spp.	Porina larvae *Wiseana* spp.	Jensen and Popay, 2004
Acremonium strictum	*H. armigera*	Jallow et al., 2004
B. bassiana	*Ostrinia nubilalis*	Lewis and Bing, 1991
B. bassiana, Clonostachys rosea	*Hypothenemus hampei*	Vega et al., 2008

Coleomegilla maculata, a predator of *O. nubilalis* eggs and larvae (Pingel and Lewis, 1996). Research on *B. bassiana* as a maize endophyte suggests that the reduced tunneling of *O. nubilalis* could be due to the presence of fungal metabolites that cause feeding deterrence or antibiosis rather than direct fungal infection. Other endophytes are mentioned in Table 3.

PROTOZOA

Protozoan diseases of insects are ubiquitous and comprise an important regulatory role in insect populations (Brooks, 1988). They are generally host specific and slow acting, most often producing chronic infections. The biology of most of the entomopathogenic protozoa is complex. They develop only in living hosts and many species require an intermediate host. Species in the Microsporidia are among the most commonly observed. Their main advantages are persistence and recycling in host populations and their debilitating effect on reproduction and overall fitness of target insects. As inundatively applied microbial control agents, only a few species have been moderately successful (Solter and Becnel, 2000). The grasshopper pathogen *Nosema locustae* is the only species that has been registered and commercially developed (Henry and Oma, 1981).

CONCLUSION

Many of the microbial based insecticides perform well *in vitro*. However, their action is negated *in vivo*. To overcome such negatives, efficient entomopathogenic strains and effective formulations are required. Other way, the actual target insect or microclimate of insect can be modified to make it susceptible to microbe. For instance, in mealybugs and wooly aphids, waxy coating prevents the microbial entry. In such a case, some materials with organic/non-polar nature which remove the upper coating of the insect to provide the entry point are useful. In Tropical countries, high temperatures influence the activity of microbial pathogens. Temperature tolerant strains from the areas with high temperatures could be adapted in other localities. Relative humidity is required for the growth of entomopathogenic fungi. Hence, good control of the insects could be achieved in rainy season where congenial humid conditions will prevail for the entomopathogen. Correspondingly, the crop canopy also influences the potency of entomopathogen. For instance, *Nomuraea rileyi* was quiet effective in crops such as groundnut and soybean for insect control with bushy canopy.

Expression of Bt in crops could be checked with available Bt strips in the market. Similarly, diagnosable tools are vital for other microbial pesticides as well. Plant metabolites such as peroxidases may hinder the action of microbial agents (Hoover et al., 1998). The inactivation can be reduced by addition of free radical scavengers such as mannitol or enzyme superoxide dismutase to baculovirus preparations (Zhou et al., 2004). Research should be directed on efficient delivery system to hit the target. It has been shown that honey bee-mediated delivery of the insect pathogen, *M. anisopliae*, increased pollen beetle control (*Meligethes* spp.) in oilseed rape (Butt et al., 1998) compared to conventional sprayers in delivering the inoculum to the pest infested flowers.

A new method for delivering viruses to target insects by using *Trichogramma* spp. as vector has been developed in China (Peng et al., 1998): *Trichogramma dendrolimi* combined with HaNPV to control the cotton bollworm (Zhu et al., 2002). The potency of microbes can be enhanced by combining with insect growth regulators. Chlorfluazuron enhanced the biological activity of AcMNPV against *S. exigua* and SlNPV against *S. litura* (Guo et al., 2007). Though there is lot of scope for microbial control, it is not comparable with chemical control strategies. Genetic engineering of microbes could fill the gap to some extent. However, farmers are interested in chemicals that show quick knock-down effect. For this, factors like enzymes and metabolites that determine pathogen virulence should be identified and used in strain selection and quality control. Advanced chemical technology could be adapted to formulate microbials as best insecticides like spinosad, a microbial based insecticide. Much emphasis should be given by concerned organization for research in development of microbials as pesticides. At the end, it should be noted that microbials with no environmental concerns effectively fits in Integrated Pest Management programs.

Conflict of Interests

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

ACKNOWLEDGEMENTS

The authors are grateful to the Department of Biotechnology, India, for the financial assistance (BT/PR13824/AGR/05/508/2010). We thank P.V.S. Prasad for the manuscript formatting.

REFERENCES

Akbar W, Lord JC, Nechols JR, Loughin TM (2005). Efficacy of *Beauveria bassiana* for Red flour beetle when applied with plant essential oils or in mineral oil and organ silicone carriers. J. Econ. Entomol. 98(3):683-688.

Anonymous (1998). United States Environmental Protection Agency, R.E.D. Facts, *Bacillus thuringiensis*, Prevention, Pesticides and Toxic substances (751 W), EPA-738-F-98-001.

Anonymous (2007). US Environmental Protection Agency (2007). Information published at web site. Info@healthgoods.com.

Askary H, Carriere Y, Belanger RR, Brodeur J (1998). Pathogenicity of the fungus *Verticillium lecanii* to aphids and powdery mildew. Biocont. Sci. Technol. 8:23-32.

Askary H, Yarmand H (2007). Development of the entomopathogenic hyphomycete *Lecanicillium muscarium* (Hyphomycetes: Moniliales) on various hosts. Eur. J. Entomol. 104:67-72.

Bahar AA, Demirbag Z (2007). Isolation of pathogenic bacteria from *Oberea linearis* (Coleptera: Cerambycidae). Biologia 62:13-18.

Bahar AA, Sezen K, Demirbağ Z, Nalçacioğlu R (2011). The relationship between insecticidal effects and chitinase activities of Coleopteran-originated entomopathogens and their chitinolytic profile. Ann. Microbiol. 62(2):647-653.

Bandani AR, Butt TM (1999). Insecticidal, anti-feedant and growth inhibitory activities of efrapeptins, metabolites of the fungus *Tolypocladium*. Biocont. Sci. Technol. 9:499-506.

Barreto MR, Loguercio LL, Valicente FH, Paiva E (1999). Biological control insecticidal activity of culture supernatants from *Bacillus thuringiensis* Berliner strains against *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae) larvae. Ann. Soc. Entomol. Brasil 28(4):675.

Bellotti AC (1999). Recent advances in cassava pest management. Annu. Rev. Entomol. 44:345-370.

Bhalla R, Dalal M, Panguluri SK, Jagadish B, Mandaokar AD, Singh AK, Kumar PA (2005). Isolation, characterization and expression of a novel vegetative insecticidal protein gene of *Bacillus thuringiensis.* FEMS Microbiol. Lett. 243:467-472.

Bonning BC, Possee RD, Hammock BD (1999). Insecticidal efficacy of a recombinant baculovirus expressing JHE-KK, a modified juvenile hormone esterase. J. Invert. Pathol. 73:234-236.

Boonphong S, Kittakoop P, Isaka M, Palittapongarnpim P, Jaturapat A, Danwisetkanjana K, Tanticharoen M, Thebtaranonth Y (2001). A new antimycobacterial, 3b-acetoxy-15a, 22-dihydroxyhopane, from the insect pathogenic fungus *Aschersonia tubulata.* Planta Med. 67:279-281.

Bowen D (1995). Characterization of a high molecular weight insecticidal protein complex produced by the entomopathogenic bacterium *Photorhabdus luminescens.* [Ph.D. thesis], University of Wisconsin, Madison

Bowen D, Blackburn M, Rocheleau T, Grutzmacher C, Ffrench-Constant RH (2000). Secreted proteases from *Photorhabdus luminescens*: Separation of the extracellular proteases from the insecticidal tc toxin complexes. Insect Biochem. Mol. Biol. 30:69-74.

Bream AS, Ghazal SA, El-Aziz ZKA, Ibrahim SY (2001). Insecticidal activity of selected actinomycetes strains against the Egyptian cotton leaf worm *Spodoptera littoralis* (Lepidoptera: Noctuidae). Mededelingen Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen Universiteit Gent. 66(2a):503-544.

Broderick NA, Raffa KF, Handelsman J (2006). Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. PNAS 103(41):15196-15199.

Broderick NA, Robinson CJ, McMahon MD, Holt J, Handelsman J, Raffa KF (2009). Contributions of gut bacteria to *Bacillus thuringiensis*-induced mortality vary across a range of Lepidoptera. BMC Biol. 7:11.

Brooks WM (1988). Entomogenous Protozoa. Handbook of Natural Pesticides, Vol. V: Microbial Insecticides, Part A. In: Ignoffo CM, Mandava NB (eds) Entomogenous Protozoa and Fungi, CRC Press, Boca Raton, FL. pp. 1-149.

Burden JP, Hails RS, Windass JD, Suner MM, Cory JS (2000). Infectivity, speed of kill, and productivity of a Baculovirus expressing the itch mite toxin txp-1 in second and fourth instar larvae of *Trichoplusia ni.* J. Invert. Pathol. 7:226-236.

Burnell AM, Stock SP (2000). *Heterorhabditis*, *Steinernema* and their bacterial symbionts - lethal pathogens of insect. Nematology 2:31-42.

Butt TM, Carreck NL, Ibrahim L, Williams IH (1998). Honey bee mediated infection of pollen beetle (*Meligethes* spp.) by the insect-pathogenic fungus, *Metarhizium anisopliae.* Biocontrol Sci. Technol. 8:533-538.

Chang JH, Choi JY, Jin BR, Roh JY, Olszewski JA, Seo SJ, O'Reilly DR, Je YH (2003). An improved baculovirus insecticide producing occlusion bodies that contain *Bacillus thuringiensis* insect toxin. J. Invert. Pathol. 84:30-37.

Chattopadhyay A, Bhatnagar NB, Bhatnagar R (2004). Bacterial Insecticidal Toxins. Crit. Rev. Microbiol. 30:33-54.

Choi JY, Wang Y, Kim YS, Kang JN, Roh JY, Woo SD, Jin BR, Je YH (2008). Insecticidal activities of recombinant *Autographa californica* nucleopolyhedrovirus containing a scorpion neurotoxin gene using promoters from *Cotesia plutellae* bracovirus. J. Asia Pac. Entomol. 1:155-159.

Clement SL, Elberson LR, Bosque-Perez NA, Schotzko DJ (2005). Detrimental and neutral effects of wild barley-Neotyphodium fungal endophyte associations on insect survival. Entomol. Exp. Appl. 114:119-125.

Companta S, Clement C, Sessitsch A (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. Soil Biol. Biochem. 42:669-678.

Daisy S, Strobel G, Ezra D, Castillo UF Baird G, Hess WM (2002). *Muscodor vitigenus* anam. sp. nov. An endophyte from *Paullinia paulliniodes.* Mycotaxon 84:39-50.

Desai JD, Banat IM (1997). Microbial production of surfactants and their commercial potential. Microbiol. Mol. Biol. Rev. 61:414-764.

Dhanasekaran D, Sakthi V, Thajuddin N, Panneerselvam A (2010). Preliminary evaluation of anopheles mosquito larvicidal efficacy of mangrove actinobacteria. Int. J. Appl. Biol. Pharm. Technol. 1(2):374-381.

Dingle J, McGee PA (2003). Some endophytic fungi reduce the density of pustules of *Puccinia recondita* f. sp. *tritici* in wheat. Mycol. Res.107:310-316.

Duchaud E, Rusniok C, Frangeul L, Buchrieser C, Givaudan A, Taourit S, Bocs S, Boursaux-Eude C, Chandler M, Charles JF, Dassa E, Derose R, Derzelle S, Freyssinet G, Gaudriault S, Medigue C, Lanois A, Powell K, Siguier P, Vincent R, Wingate V, Zouine M, Glaser P, Boemare N, Danchin A, Kunst F (2003). The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens.* Nat. Biotechnol. 21:1307-1313.

Elmi AA, West CP, Robbins RT, Kirkpatrick TL (2000). Endophyte effects on reproduction of a root-knot nematode (*Meloidogyne marylandi*) and osmotic adjustment in tall fescue. Grass Forage Sci. 55:166-172.

Fang J, Xu XL, Wang P, Zhao JZ, Shelton AM, Cheng J, Feng MG, Shen ZC (2007). Characterization of chimeric *Bacillus thuringiensis* Vip3 toxins. Appl. Environ. Microbiol. 73:956-961.

FAO (2012). http://www.fao.org/news/story/en/item/131114/icode

Faria M de, Wraight SP (2001). Biological control of *Bemisia tabaci* with fungi. Crop Prot. 20(9):767-778.

Freed S, Feng-Liang J, Naeem M, Shun-Xiang R, Hussian M (2012). Toxicity of proteins secreted by Entomopathogenic fungi against *Plutella xylostella* (Lepidoptera: Plutellidae). Int. J. Agric. Biol. 14(2):291-295.

French-Constant RH, Bowen DJ (2000). Novel insecticidal toxins from nematode-symbiotic bacteria. Cell. Mol. Life Sci. 57:828-833.

Gadelhak GG, EL-Tarabily KA, AL-Kaabi FK (2005). Insect control using chitinolytic soil actinomycetes as biocontrol agents. Int. J. Agric. Biol.

7(4):627-633.
George J, Laurence GR, Frederick MA (2000). Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. J. Bacteriol. 182(13):3843.
Goettel MS, Hajek AE (2001). 'Evaluation of non-target effects of pathogens used for management of Arthropods'. In: Wajnberg E, Scott JK, Quimby PC, (eds) Evaluating indirect ecological effects of Biological Control, CABI Press, Wallingford, UK. pp. 81-97.
Gopalakrishnan S, Ranga Rao GV, Humayun P, Rameshwar Rao V, Alekhya G, Simi J, Deepthi K, Sree Vidya M, Srinivas V, Mamatha L, Rupela O (2011). Efficacy of botanical extracts and entomopathogens on control of *Helicoverpa armigera* and *Spodoptera litura.* Afr. J. Biotechnol. 10(73):16667-16673.
Govindarajan M, Jebanesan A, Reetha D (2005). Larvicidal effect of extracellular secondary metabolites of different fungi against the mosquito *Culex quinquefasciatus* Say. Trop. Biomed. 22(1):1-3.
Gramkow AW, Perecmanis S, Sousa RLB, Noronha EF, Felix CR, Nagata T, Ribeiro BM (2010). Insecticidal activity of two proteases against *Spodoptera frugiperda* larvae infected with recombinant baculoviruses. Virology J. 7:143.
Grzywacz D, Richards A, Rabindra RJ, Saxena H, Rupela OP (2005). Efficacy of biopesticides and natural plant products for *Heliothis/Helicoverpa* control. In: Sharma HC(ed) Heliothis/ Helicoverpa Management-Emerging Trends and Strategies for Future Research, Oxford and IBH Publishing Co. Pvt. Ltd. New Delhi. pp. 371-389.
Guo HF, Fang JC, Liu BS, Wang JP, Zhong WF, Wan FH (2007). Enhancement of the biological activity of nucleopolyhedrovirus through disruption of the peritrophic matrix of insect larvae by chlorfluazuron. Pest Manag. Sci. 63:68-74.
Harman GE (2011). *Trichoderma* - not just for biocontrol anymore. Phytoparasitica 39:103-108.
Henry JE, Oma EA (1981). Pest control by *Nosema locustae,* a pathogen of grasshoppers and crickets. In: Burges HD (ed) Microbial Control of Pests and Plant Diseases 1970-1980, Academic Press, London. pp. 573-586.
Herbert AK (2010). The spinosyn family of insecticides: realizing the potential of natural products research. J. Antibiot. 63:101-111.
Hoell IA, Klemsdal SS, Vaaje-Kolstad G, Horn SJ, Eijsink VGH (2005). Overexpression and characterization of a novel chitinase from *Trichoderma atroviride* strain. Biochim. Biophys. Acta. 1748:180-190.
Hoover K, Kishida KT, DiGiorgio LA, Workman J, Alaniz SA, Hammock BD, Duffey SS (1998). Inhibition of baculoviral disease by plant-mediated peroxidase activity and free radical generation. J. Chem. Ecol. 24:1949-2001.
Hu QB, Ren SX, An XC, Qian MH (2007). Insecticidal activity influence of destruxins on the pathogenicity of *Paecilomyces javanicus* against *Spodoptera litura.* J. Appl. Entomol. 131:262-268.
Huamei L, Sheng Qf, Yongxia W, Wenjun L, Jie Z (2008). Insecticidal action of Quinomycin A from *Streptomyces* sp. KN-0647 isolated from a forest soil. World J. Microbiol. Biotechnol. 24: 2243-2248.
Hussain AA, Mostafa SA, Ghazal SA, Ibrahim SY (2002). Studies on antifungal antibiotic and bioinsecticidal activities of some actinomycete isolates. Afr. J. Mycol. Biotechnol. 10:63-80.
Ignoffo CM (1981). The fungus *Nomuraea rileyi* as a microbial insecticide: fungi. In: Burges, HD (ed) Microbial Control of Pests and Plant Diseases, Academic Press, London, UK. pp. 513-538.
Ignoffo CM, Garcia C, Zuidema D, Vlak JM (1995). Relative *in vivo* activity and simulated sunlight-UV stability of inclusion bodies of a wild-type and an engineered polyhedral envelope negative isolate of the nucleopolyhedrosis virus of *Autographa californica.* J. Invert. Pathol. 66:212-213.
Imai N, Ses A, El-Singabi NR, Iwanaga M, Matsumoto S, Iwabuchi K, Maeda S (2000). Insecticidal effects of a recombinant baculovirus expressing a scorpion toxin LqhiT2. J. Seric. Sci. Jpn. 69:197-205.
Jallow MFA, Dugassa-Gobena D, Vidal S (2004). Indirect interaction between and unspecialized endophytic fungus and a polyphagous moth. Basic Appl. Ecol. 5:183-191.
Jensen JG, Popay AHJ (2004). Perennial ryegrass infected with AR 37 endophyte reduces survival of porina larvae. N. Z. Plant Prot. 57:323-328.
Keller S (2000). Use of *Beauveria brongniartii* in Switzerland and its acceptance by farmers. Bull. OILB/SROP 23(8):67-71.
Kershaw MJ, Moorhouse ER, Bateman R, Reynolds SE, Charnley AK (1999). The role of destruxins in the pathogenicity of *Metarhizium anisopliae* for three species of insect. J. Invert. Pathol. 74:213-223.
Kim JJ, Goettel MS, Gillespie DR (2007). Potential of *Lecanicillium* species for dual microbial control of aphids and the cucumber powdery mildew fungus *Sphaerotheca fuliginea.* Biol. Control 40:327-332.
Kim JJ, Goettel MS, Gillespie DR (2008). Evaluation of *Lecanicillium longisporum* Vertalec for simultaneous suppression of cotton aphid, *Sphaerotheca fuliginea*, on potted cucumbers. Biol. Control 45:404-409.
Kim JJ, Lee MH, Yoon CS, Kim HS, Yoo JK, Kim KC (2002). Control of cotton aphid and greenhouse whitefly with a fungal pathogen. J. Nat. Inst. Agri. Sci. Technol. pp. 7-14.
Klingen I, Eilenberg J, Meadow R (1998). Insect pathogenic fungi from northern Norway baited on *Delia floralis* (Diptera, Anthomyiidae) and *Galleria mellonella* (Lepidoptera, Pyralidae). IOBC wprs Bull. 21:121-124.
Konstantopoulou MA, Mazomenos BE (2005). Evaluation of *Beauveria bassiana* and *B. brongniartii* strains and four wild-type fungal species against adults of *Bactrocera oleae* and *Ceratitis capitata*. Biol. Control 50:293-305.
Kroiss J, Kaltenpoth MB, Schneider B, Schwinger M, Hertweck C, Maddula R, Strohm E, Svatos A (2010). Symbiotic Stereptomycetes provide antibiotic combination prophylaxis for wasp offspring. Nat. Chem. Biol. 6:261-263.
Kumari V, Singh NP (2009). *Spodoptera litura* nuclear polyhedrosis virus (NPV-S) as a component in Integrated Pest Management (IPM) of *Spodoptera litura* (Fab.) on cabbage. J. Biopestic. 2:84-86.
Kuzina LV, Peloquin JJ, Vacek DC, Miller TA (2001). Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies *Anastrepha ludens* (Diptera: Tephritidae). Curr. Microbiol. 42:290-294.
Lacey LA, Neven LG (2006). The potential of the fungus, *Muscodor albus*, as a microbial control agent of potato tuber moth (Lepidoptera: Gelechiidae) in stored potatoes. J. Invert. Pathol. 91:195-198.
Lau GW, Goumnerov BC, Walendziewicz CL, Hewitson J, Xiao W, Mahajan-Miklos S, Tompkins RG, Perkins LA, Rahme LG (2003). The *Drosophila melanogaster* toll pathway participates in resistance to infection by the gram-negative human pathogen *Pseudomonas aeruginosa.* Infect. Immun. 71:4059-4066.
Lewis LC, Berry EC, Obrycki JJ, Bing LA (1996). Aptness of insecticides (*Bacillus thuringiensis* and carbofuran) with endophytic *Beauveria bassiana*, in suppressing larval populations of the European corn borer. Agric. Ecosyst. Environ. 57:27-34.
Lewis LC, Bing LA (1991). *Bacillus thuringiensis* Berliner and *Beauveria bassiana* (Balsamo) Vuillemin for European corn borer control: Program for immediate and season long suppression. Can. Entomol. 123:387-393.
Lewis LC, Bruck DJ, Gunnarson RD, Bidne KG (2001). Assessment of plant pathogenicity of endophytic *Beauveria bassiana* in Bt transgenic and non-transgenic corn. Crop Sci. 41:1395-1400.
Li Z, Wang Z, Peng G, Yin Y, Zhao H, Cao Y, Xia Y (2007). Regulation of extracellular acid phosphatase biosynthesis by culture conditions in entomopathogenic fungus *Metarhizium anisopliae* strain. Ann. Microbiol. 57(4):565-570.
Li ZZ, Li CR, Huang B, Meizhen MZ (2001). Discovery and demonstration of the teleomorph of *Beauveria bassiana* (Bals.) Vuill. an important entomogenous fungus. Chin. Sci. Bull. 46(9):751-753.
Liu F, Yang W, Ruan L, Sun M (2013). A *Bacillus thuringiensis* host strain with high melanin production for preparation of light-stable biopesticides. Ann. Microbiol. 63(3):1131-1135.
Mathew SO, Sandhu SS, Rajak RC (1998). Bioactivity of *Nomuraea rileyi* against *Spilosoma obliqua*: effect of dosage, temperature and relative humidity. J. Ind. Bot. Soc. 77:23-25.
Mazet I, Hung SY, Boucias DG (1995). Hirsutellin A, a toxic protein produced *in vitro* by *Hirsutella thompsonii.* J. Invert. Pathol. 64:200-207.
Moscardi F (1999). Assessment of the applications of baculoviruses for control of Lepidoptera. Annu. Rev. Entomol. 44:257-289.
Mostakim M, Soumya E, Mohammed IH, Ibnsouda SK (2012). Biocontrol

potential of a *Pseudomonas aeruginosa* strain against *Bactrocera oleae.* Afr. J. Microbiol. Res. 6(26):5472-5478.
Muratoglu H, Kati H, Demirbag Z Sezen K (2009). High insecticidal activity of *Leclercia adecarboxylata* isolated from *Leptinotarsa decemlineata* (Col.: Chrysomelidae). Afr. J. Biotechnol. 8(24):7111-7115.
Muratoglu H, Nalcacioglu, Demibag Z (2010). Transcriptional and structural analyses of *Amsacta moorei* entomopoxvirus protein kinase gene (AMV197, *pk*). Ann. Microbiol. 60:523-530
Murty MG, Srinivas G, Sekar V (1994). Production of a mosquitocidal exotoxin by a *Pseudomonas fluorescens* strain. J. Invert. Pathol. 64:68-70.
Nishiwaki H, Nakashima K, Ishida C, Kawamura T, Matsuda K (2007). Cloning, functional characterization, and mode of action of a novel insecticidal poreforming toxin, sphaericolysin, produced by *Bacillus sphaericus.* Appl. Environ. Microbiol. 73:3404-3411.
Nunez E, Iannacone J, Gomez H (2008). Effect of two entomopathogenic fungi in controlling *Aleurodicus cocois* (Curtis 1846) (Hemiptera: Aleyrodidae). Chil. J. Agric. Res. 68(1):21-30.
Oliveira I, Pereira A, Bento A, Baptista P (2011). Viability of *Beauveria bassiana* isolates after storage under several preservation methods. Ann. Microbiol. 61:339-344.
Osborn F, Berlioz L, Vitelli-Flores J, Monsalve W, Dorta B, Lemoine VR (2002). Pathogenic effects of bacteria isolated from larvae of *Hylesia metabus* Crammer (Lepidoptera: Saturniidae). J. Invert. Pathol. 80:7-12.
Patil CD, Borase HP, Salunke BK, Patil SV (2013). Alteration in *Bacillus thuringiensis* toxicity by curing gut flora: novel approach for mosquito resistance management. Parasitol. Res. 112:3283-3288.
Peng HY, Chen XW, Jiang Y (1998). Controlling *Dendrolimus punctatus* with *Trichogramma dendrolimi* carrying cytoplasmic polyhedrosis virus. Chin. J. Biol. Control 14:111-114.
Pinedo FJR, Moscardi F, Luque T, Julie A, Olszewski Bergmann MR (2003). Inactivation of the ecdysteroid UDP-glucosyltransferase (egt) gene of *Anticarsia gemmatalis* nucleopolyhedrovirus (AgMNPV) improves its virulence towards its insect host. Biol. Control 27:336-344.
Pingel RL, Lewis LC (1996). The fungus *Beauveria bassiana* (Balsamo) Vuillemin in a corn ecosystem: Its effect on the insect predator *Coleomegilla maculata* De Geer. Biol. Control 6:137-141.
Pitterna T, Cassayre J, Huter O (2009). New Ventures in the Chemistry of Avermectins. Bioorg. Med. Chem. 17:4085-4095.
Prikhod'ko Prikhod'ko GG, Popham HJR, Felcetto TJ, Ostlind DA, Warren VA, Smith MM, Garsky VM, Warmke JW, Cohen CJ, Miller LK (1998). Effects of simultaneous expression of two sodium channel toxin genes on the properties of baculoviruses as biopesticides. Biol. Control 12:66-78.
Priyanka J, Srivastava N, Prakash S (2001). *Chrysosporium tropicum* efficacy against *Anopheles stephensi* larvae in the laboratory. J. Am. Mosq. Control Assoc. 17:127-130.
Purcell JP, Greenplate JT, Jennings MG, Ryerse JS, Pershing JC, Sims SR, Prinsen MJ, Corbin DR, Tran M, Sammons RD, Stonard RJ (1993). Cholesterol oxidase: a potent insecticidal protein active against boll weevil larvae. Biochem. Biophys. Res. Commun.196:1406-1413.
Quesada-Moraga E, Carrasco-Diaz JA, Santiago-Alvarez C (2006). Insecticidal and anti-feedant activities of proteins secreted by entomopathogenic fungi against *Spodoptera littoralis* (Lep., Noctuidae). J. Appl. Entomol. 130:442-452.
Quesada-Moraga E, Vey A (2004). Bassiacridin, a protein toxic for locusts secreted by the entomopathogenic fungus *Beauveria bassiana.* Mycol. Res. 108:441-452.
Rabindra RJ, Singh A, Saxena H, Chandish BR (2005). Biological control of insect pests and diseases in food legumes. In: International Food Legumes Research Conference, Abstracts-4, October 18 -22, New Delhi. pp. 25-26.
Rajak RC, Sandhu SS, Mukherjee S, Kekre S, Gupta A (1991). Natural outbreak of *Nomuraea rileyi* on *Junonia orithyia.* J. Biol. Control 5(2):123-124.
Ranga Rao GV, Rupela OP, Rameshwar Rao V, Reddy YVR (2007). Role of biopesticides in crop protection: Present status and future prospects. Indian J. Plant Prot. 35(1):1-9.
Revathi N, Ravikumar G, Kalaiselvi M, Gomathi D, Uma C (2011). Pathogenicity of three entomopathogenic fungi against *Helicoverpa armigera*. J. Plant Pathol. Microbiol. 2:114.
Sahayaraj K, Borgio JF (2010). Virulence evaluation of entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) Sorokin (Deuter.: Hyphomycetes) on seven insect pests. Indian J. Agric. Sci. 44:195-200.
Salgado VL (1998). Studies on the mode of action of Spinosad: Insect symptoms and physiological correlates. Pestic. Biochem. Physiol. 60:91-102.
Sandhu SS, Rajak RC, Hasija SK (2000). Potential of entomopathogens for the biological management of medically important pest: Progress and prospect. In: Glimpses in Plant Sciences. pp. 110-117.
Sandra WW, Douglas IG (2004). Microorganisms associated with field-collected *Chrysoperla rufilabris* (Neuroptera: Chrysopidae) adults with emphasis on yeast symbionts. Biol. Control 29:155-168.
Seleena P, Lee HL (1994). Insecticidal activity of a Malaysian isolate of *Aspergillus niger*. Asean J. Sci. Technol. Dev. 11(2):47-53.
Sellami S, Jamoussi K, Dabbeche E, Jaoua S (2011). Increase of the *Bacillus thuringiensis* secreted toxicity against lepidopteran larvae by homologous expression of the vip3LB gene during sporulation stage. Curr. Microbiol. 63:289-294.
Seo JH, Yeo JS, Cha HJ (2005). Baculoviral Polyhedrin-*Bacillus thuringiensis* toxin fusion protein: A Protein-based bio-insecticide expressed in *Escherichia coli.* Biotechnol. Bioeng. 92(2):166-172.
Sergeant M, Baxter L, Jarrett P, Shaw E, Ousley M, Winstanley C, Alun J, Morgan W (2006). Identification, typing, and insecticidal activity of *Xenorhabdus* isolates from entomopathogenic nematodes in United Kingdom soil and characterization of the *xpt* toxin loci. Appl. Environ. Microbiol. 72(9):5895-5907.
Sevim A, Demirbag Z, Demirturk I (2010). A new study on the bacteria of *Agrotis segetum* Schiff. (Lepidoptera: Noctuidae) and their insecticidal activities. Turk. J. Agric. For. 34:333-342.
Sezen K, Demir I, Demirbag Z (2005). Investigations on bacteria as a potential biological control agent of summer chafer *Amphimallon solstitiale* L. (Coleoptera: Scarabaeidae). J. Microbiol. 43:463-468.
Shakeri J, Foster HA (2007). Proteolytic activity and antibiotic production by *Trichoderma harzianum* in relation to pathogenicity to insects. Enzyme Microb. Technol. 40(5):961-968.
Sharaf EF (2005). A potent chitonylitic activity of *Alternaria alternata* isolated from Egyptian black sand. Pol. J. Microbiol. 54(2):145-151.
Sharma S, Waterfield N, Bowen D, Rocheleau T, Holland L, James R, Ffrench-Constant R (2002). The lumicins: novel bacteriocins from *Photorhabdus luminescens* with similarity to the uropathogenic-specific protein (USP) from uropathogenic *Escherichia coli.* FEMS Microbiol. Lett. 214:241-249.
Snyder DE, Meyer J, Zimmerman AG, Qiao M, Gissendanner SJ, Cruthers LR, Slone RL, Young DR. (2007). Preliminary studies on the effectiveness of the novel pulicide, spinosad, for the treatment and control of fleas on dogs. Vet. Parasitol. 150(4):345-351.
Solter LF, Becnel JJ (2000). Entomopathogenic microsporida. Field Manual of Techniques in Invertebrate Pathology. In: Lacey LA, Kaya HK, (eds) Application and Evaluation of Pathogens for Control of Insects and Other Invertebrate Pests. Kluwer Academic, Dordrecht. pp. 231-254.
Srinivasa M, Jagadeesh Babu CS, Anitha CN, Girish G (2008). Laboratory evaluation of available commercial formulations of HaNPV against *Helicoverpa armigera* (Hub.). J. Biopestic. 1:138-139.
Stinson AM, Zidack NK, Strobel GA, Jacobsen BJ (2003). Mycofumigation with *Muscodor albus* and *Muscodor roseus* for control of seedling disease of sugar beet and Verticillium wilt of eggplant. Plant Dis. 87:1349-1354.
Strasser H, Vey A, Butt TM (2000). Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium*, *Tolypocladium* and *Beauveria* species? Biocon. Sci. Tech. 10:717-735.
Sundarapandian S, Sundaram MD, Tholkappian P, Balasubramanian V (2002). Mosquitocidal properties of indigenous fungi and actinomycetes against *Culex quinquefasciatus* Say. J. Biol. Control 16:89-91.
Szolajska E, Poznanski J, Ferber ML, Michalik J, Gout E, Fender P, Bailly I, Dublet B, Chroboczek J (2004). Poneratoxin, a neurotoxin

from ant venom.Structure and expression in insect cells and construction of a bio-insecticide. Eur. J. Biochem. 271:2127-2136.
Thakur R, Sandhu SS (2010). Distribution, occurrence and natural invertebrate hosts of indigenous entomopathogenic fungi of Central India. Indian J. Microbiol. 50(1):89-96.
Thiery I, Frachon E (1997). Identification, isolation, culture and preservation of enthomopathogenic bacteria. In: Lacey LA (ed) Manual of Techniques in Insect Pathology, Academic Press, London. pp. 55-73.
Tuan SJ, Hou RF, Lee CF, Chao YC (2007). High level production of polyhedra in a scorpion toxin containing recombinant baculovirus for better control of insect pests. Bot. Stud. 48:273-281.
Vandermeer J, Perfecto I, Liere H (2009). Evidence for hyperparasitism of coffee rust *Hemileia vastatrix* by the entomogenous fungus, *Lecanicillium lecanii*, through a complex ecological web. Plant Pathol. 58:636-641.
Vega FE, Posada F Aime MC, Pava-Ripoll M, Infante F, Rehner SA (2008). Entomopathogenic fungal endophytes. Biol. Control 46:72-82.
Vey A, Hoagland RE, Butt TM (2001). Toxic metabolites of fungal control agents. In: Butt TM, Jackson C, Magan N (eds). Fungi as Biocontrol Agents, CAB International, New York. pp. 311-346.
Vimala Devi PS (2001). Prospects of using *Nomuraea rileyi* for the management of crop pests. In: Rabindra RJ, Kennedy JS,Sathiah N, Rajasekaran B, Srinivasan MR (eds). Microbial Control of Crop Pests, Graphic Skill Publisher, Coimbatore. pp. 80-94.
Wicklow DT, Roth S, Deyrup ST, Gloer JB (2005). A protective endophyte of maize: *Acremonium zeae* antibiotics inhibitory to *Aspergillus flavus* and *Fusarium verticillioides.* Mycolog. Res. 109:610-618.
Yankouskaya A (2009). Application of biological insecticide Pecilomicine-B for greenhouse pest control. Scientific works of the Lithuanian Institute of Horticulture and Lithuanian University of Agriculture. Sodininkystė Ir Daržininkystė 28(3):249-258.
Yu CG, Mullins MA, Warren GW, Koziel MG, Estruch JJ (1997). The *Bacillus thuringiensis* vegetative insecticidal protein Vip3A lyses midgut epithelium cells of susceptible insects. Appl. Environ. Microbiol. 63:532-536.
Zhang GY, Sun XL, Zhang ZX, Zhang ZF, Wan FF (1995). Production and effectiveness of the new formulation of *Helicoverpa* virus pesticide-emulsifiable suspension. Virol. Sin. 10:242-247.
Zhang J, Zhao J, Li D, Liu S, Li L, Sun Q, Huang M, Yang Z (2009). Cloning of the gene encoding an insecticidal protein in *Pseudomonas pseudoalcaligenes*. Ann. Microbiol. 59(1):45-50
Zhou MZ, Sun HC, Hu ZH, Sun XL (2004). SOD enhances infectivity of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrosis against *Helicoverpa. armigera* larvae. Virol. Sin. 18:506-507.
Zhu C, Ruan L, Peng D, Yu Z, Sun M (2006). Vegetative insecticidal protein enhancing the toxicity of *Bacillus thuingiensis* subsp *kurstaki* against *Spodoptera exigua.* Lett. Appl. Microbiol. 42:109-114.
Zhu JQ, Lei CL, Peng HY (2002). Studies on the effect of *Trichogramma dendrolimi* carrying NPV to suppress *Helicoverpa armigera.* Nat. Enemies Insects 24:20-25.

academicJournals

Vol. 13(26), pp. 2641-2647, 25 June, 2014
DOI: 10.5897/AJB2014.13622
Article Number: A2743AB45609
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Larvicidal efficacy of *Jatropha curcas* L. (*Euphorbiaceae*) leaf and seed aqueous extracts against *Culex pipiens* L.

FATNASSI Bassem[1], KHOUJA Med Larbi [2] and EL FERCHICHI OUARDA Hela[1, 2]*

[1]Department of Biology, faculty of sciences of Bizerte, University of Carthage, Tunisia.
[2]National Institute for Research in Rural Engineering, Water and Forests, Tunisia.

Received 10 January, 2014; Accepted 16 June, 2014

Culex **mosquitoes play a vital role in transmitting pathogens which continue to have a harmful impact on human beings. Indeed, Innovative vector control strategy like use of plant extracts as alternative sources of insecticidal and larvicidal agents against vector-borne diseases has become unavoidable. In this context, the purpose of the present search was to explore the larvicidal properties of *Jatropha curcas* L. leaf and seed extracts against *Culex pipiens* L. The larvicidal activity was evaluated in eight different provenances recently introduced in Tunisia (Tanzania (ARU), Mozambique (MOZ), Surinam (SUR) and Brazil represented by five provenances: Paraná (PAR), Norte de Minas (NMB), Mato Grosso (MGB), Regiao Sureste (RSB) and Vale do Fequitinhonha (VFB). The assessment of larval activity showed after 24 h of exposure, 100% mortality for aqueous seed extract and between 60 and 100% for aqueous leaf extract according to provenances. Highest mortality is observed at 1 mg/ml against *C. pipiens* L with LC_{50} values of 0.49 and 0.5% for aqueous seed extract and leaf extract, respectively. Commonly, the mortality increase with the increase in concentration of each extract. However, the inhibitory effect of seeds extract on *C. pipiens* was more pronounced than that of leaves. These results suggest that the aqueous seed and leaf extracts of *J. curcas* have the potential to be used as an ideal eco-friendly compound for the control of hurtful mosquito larvae.**

Key words: Aqueous extract, *Culex pipiens* L., *Jatropha curcas* L., larvicidal activity.

INTRODUCTION

The species *Jatropha curcas* L. is a drought resistant shrub of the family *Euphorbiaceae*, which is predominant in Central America and today is found throughout the world in the tropics (Chavan et al., 2014). Different extracts of *J. curcas* seeds, leaves, stem and bark were used as an antiseptic, diuretic, purgative, larvicide as well as for treating cancer, gout, and skin diseases (Dalziel, 1955; Duke, 1985, 1988; Kaushik and Kumar, 2004). Many studies have been conducted on the genus *Jatropha* covering various aspects as bioactivity to insect pests of stored products (Silva et al., 2012), this property was confirmed after toxicity tests that were realized on

*Corresponding author. E-mail: helaelferchichi.ouarda@gmail.com. Tel: +216 72 591 906. Fax: +216 72 590 566.

the pests of the stocks of corn and bean seeds and the results have been spectacular, since damages in seeds were reduced to 10% (Solsoloy and Solsoloy, 1997). The aqueous extracts of bark and leaves have a larvicidal activity against mosquito species *Aedes aegypti* L. and *Culex quinquefasciatus* (Rahuman et al., 2008). The linoleic acid present in the composition of the seed oil is used in the treatment of eczema and other skin diseases (Heller, 1996). The leaf juice is an inhibitor of watermelon mosaic virus (Tewari and Shukla, 1982) and also has a human blood coagulant activity (Osoniyi and Onajobi, 2003). Roots showed an anti-inflammatory activity, the application of the powder paste spread over the inflamed part or the injection of methanolic extracts by oral or cutaneous way reduced enormously the inflammation (Mujumdar and Misar, 2004). In addition, the curcin, which is a main component of crude oil, is used in very low concentrations, as a cytotoxic agent in cancer cells; the protein that is responsible can inhibit the spread of tumor cells (Luo et al., 2006). Hence natural plant products may be a possible option to synthetic substances, as they are efficient and friendly with environment (Shaalan et al., 2005). Due to the favorable conditions for the cultivation of *J. curcas* in Tunisia, there is an increasing interest in the study of this plant and it becomes attractive as alternative mosquito larvae control agent because *J. curcas* extracts show no harmful sequel on the environment. Therefore, this encourages us to undertake a study of the larvicidal activity of seeds and leaves aqueous extracts of *J. curcas*.

MATERIALS AND METHODS

Plant material

The leaves and seeds of *J.* curcas L. were collected from the region of Nabeul (Tunisia) between April and September 2010. Plant material belongs to eight different origins. The sources are Tanzania (ARU), Mozambique (MOZ), Surinam (SUR) and five Brazilien provenances: Paraná (PAR), Norte de Minas (NMB), Mato Grosso (MGB), Regiao Sureste (SNR) and Vale do Fequitinhonha (VFB).

Animal material

Larvae subjected to toxicity tests were from larval habitat of mosquitoes untreated, located in rural areas of Mjez el bab (North West Tunisia). They were identified by Dr. Bejaoui Mustafa in Faculty of Sciences of Bizerta, according to Brunches et al. (2000). They were maintained at ambient rearing conditions in the National Institute for Research in Rural Engineering Water and Forestry. For the bioassays, only larval stage 4 was used and all tests were conducted at room temperature.

Preparation of aqueous extracts

Leaves were chapped, dried and powdered. Seeds were peeled and crushed using a mortar until having a kind of paste. Then, 100 mg of plant material was diluted in 1000 ml of distillated water previously heated to boiling. The aqueous solution was placed under magnetic stirring for 30 min. Finally, the mixture was filtered using a Whatman paper (3 MM). The recovered filtrate represents an initial stock solution with a concentration equal to 0.1 g/1000 ml.

Estimated quantity of dry residue

In order to give a more significance to the quantities of plant material soluble in aqueous extracts, they were concentrated under reduced pressure at 40°C using a rotary evaporator for 48 h, until a dry residue was obtained which quantity is expressed in mg. This helps to express the lethal concentrations of soluble dry residues in water in mg / ml.

Toxicity tests

From the initial extract (stock solution) of *J. curcas* L. seeds and leaves, and water of larvae sites, concentrations of 0.1, 0.2, 0.5 and 1 mg/ml were prepared. The tests were performed in 9 cm Petri dishes diameter, each containing 20 ml of solution and 10 mosquito larvae of *C. pipiens* of the same caliber (L4 stage). The same number of larvae was placed in a control Petri dish containing 20 ml of larvae water breeding sites. Three repetitions were performed for each concentration as well as for the control. The larvae were considered dead if they were immobile and unable to reach the water surface (Macedo et al., 1997). Mortality response was noted after exposure of 1, 2, 4, 6, 12, and 24 h, and the mortality percentage was reported from the average of three replicates.

Determination of lethal concentrations (LC_{50})

The estimates of LC_{50} were determined after 24 h of exposure using the software Spearman- Kaber (Hamilton et al., 1977).

Statistical analysis

A general linear model (ANOVA) analysis was used to determine the effect of seeds and leaves origin and the concentration of the aqueous extracts on mortality data recorded after 1, 2, 6, 12 and 24 h of treatment of the larvae. Differences between mean values were compared using the Duncan Multiple Range Test (5%) by SAS (1990), version 6.12.

RESULTS AND DISCUSSION

Evaluation of the mortality of larvae of *C. pipiens* L. exposed to aqueous seed extract of *J. curcas* L.

After 12 h of exposure, most provenances exhibited 100% mortality at 1 mg /ml except the provenances of Vale do Fequitinhonha (Brazil) and Surinam with a mortality of 76.7 and 80% respectively (Figure 1). After 24 h, the totality of provenances showed 100% mortality at 1 mg / ml and also at a concentration of 0.5 mg /ml (Figure 2). It should be noted that the Mozambique population showed a high toxicity since we obtained 100% mortality after 12 h of exposure to only a concentration of 0.2 mg /ml, while the two populations Vale do Fequitinhonha (Brazil) and Suriname showed a low toxicity, since the mortality rate after 24 h was about 96.7% for 1 mg /ml. Furthermore, the results of the

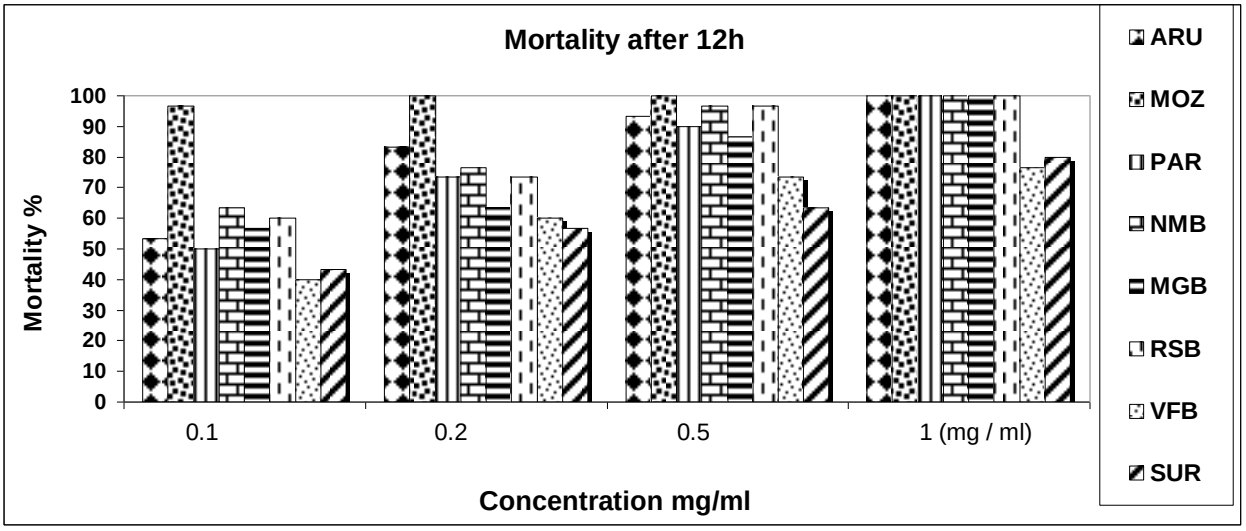


Figure 1. Mortality after 12 h of exposure of larvae to different concentrations (1 to 10%) of seed aqueous extracts.

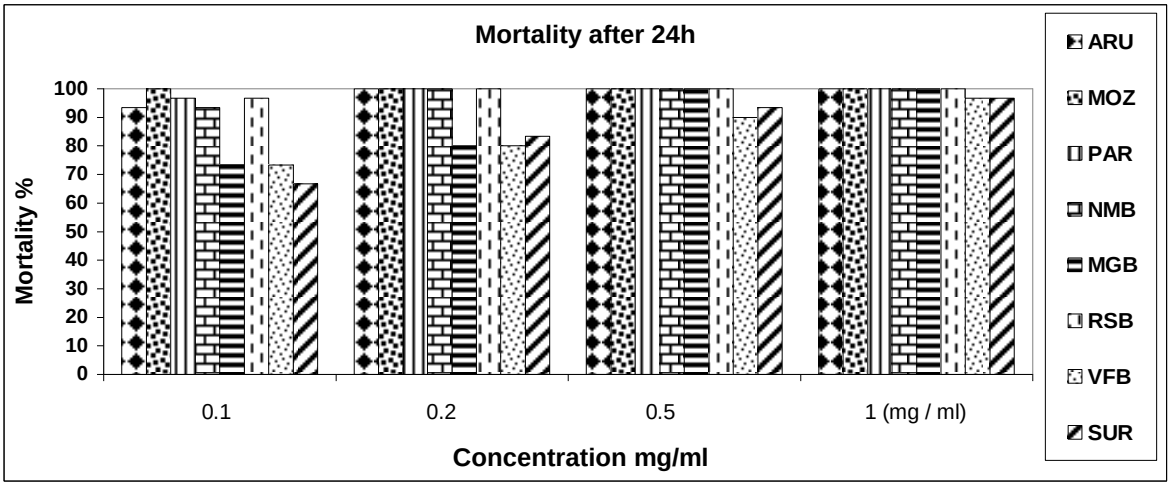


Figure 2. Mortality after 24 h of exposure of larvae to different concentrations (1 to 10%) of seed aqueous extracts.

larvicidal activity of seeds aqueous extract were subjected to analysis of variance which highlighted significant differences of larvae mortality rate between the provenances and among concentrations (Table 1). This reveals that the seeds aqueous extract is responsible for the susceptibility of *Culex pipiens* larvae. Goel et al. (2007) and Makkar et al. (1997) have shown that the higher larvicidal activity of *J. curcas* seeds extract indicate that toxic phorbol esters and other compounds are highly accumulated in seeds, rather than in other parts of the plant including the leaves.

Evaluation of the mortality of larvae of *C. pipiens* L. exposed to aqueous leaf extract of *J. curcas* L.

After the exposure of *C. pipiens* larvae to leaves aqueous extracts, it was found that after 1 h, majority of provenances present a low mortality didn't exceed 3.33% in the case of Vale do Fequitinhonha (Brazil) and Surinam, and 13.33% in the case of Paraná (Brazil) and Mato Grosso (Brazil). In both the provenances of Mozambique and Norte de Minas (Brazil), we recorded a high mortality rate which reaches 40% (Figure 3). On the other hand, after 12 h of exposure, the mortality rate reached 100% for 1 mg /ml concentration in both the provenances of Mozambique and Norte de Minas (Brazil), whereas in other provenances didn't exceed 90% (Figure 4). After 24 h, the mortality rate reached 100% only in the Mozambique, Norte de Minas (Brazil) and Regiao Sureste (Brazil) provenances. Other provenances: Tanzania, Paraná (Brazil), Mato Grosso (Brazil) and Suriname showed a low toxicity. The population of Vale do Fequitinhonha (Brazil) showed the

Table 1. Variance analyses results of larvicidal activity of seed and leaf aqueous extracts of *Jatropha curcas* L. (F and P values, for the significance of the differences)

Time (h)	Variation	Seed		Leaf	
		F	P	F	P
1	Provenance	3.57	0.0022	26.38	<0.0001
	Concentration	30.22	<0.0001	67.84	<0.0001
	Prov x Conc	2.08	0.006	6.26	<0.0001
2	Provenance	40.51	<0.0001	33.73	<0.0001
	Concentration	94.06	<0.0001	112.83	<0.0001
	Prov x Conc	4.22	<0.0001	3.06	<0.0001
4	Provenance	29.00	<0.0001	49.38	<0.0001
	Concentration	205.59	<0.0001	151.24	<0.0001
	Prov x Conc	2.76	0.002	3.98	<0.0001
6	Provenance	14.37	<0.0001	71.57	<0.0001
	Concentration	217.98	<0.0001	243.7	<0.0001
	Prov x Conc	3.14	<0.0001	5.01	<0.0001
12	Provenance	34.29	<0.0001	109.93	<0.0001
	Concentration	804.59	<0.0001	476.32	<0.0001
	Prov x Conc	5.02	<0.0001	7.59	<0.0001
24	Provenance	20.90	<0.0001	134.69	<0.0001
	Concentration	1803.36	<0.0001	716.4	<0.0001
	Prov x Conc	4.83	<0.0001	10.35	<0.0001

$F_{theoric}$ = 1.83 (5%); 2.32 (1%).

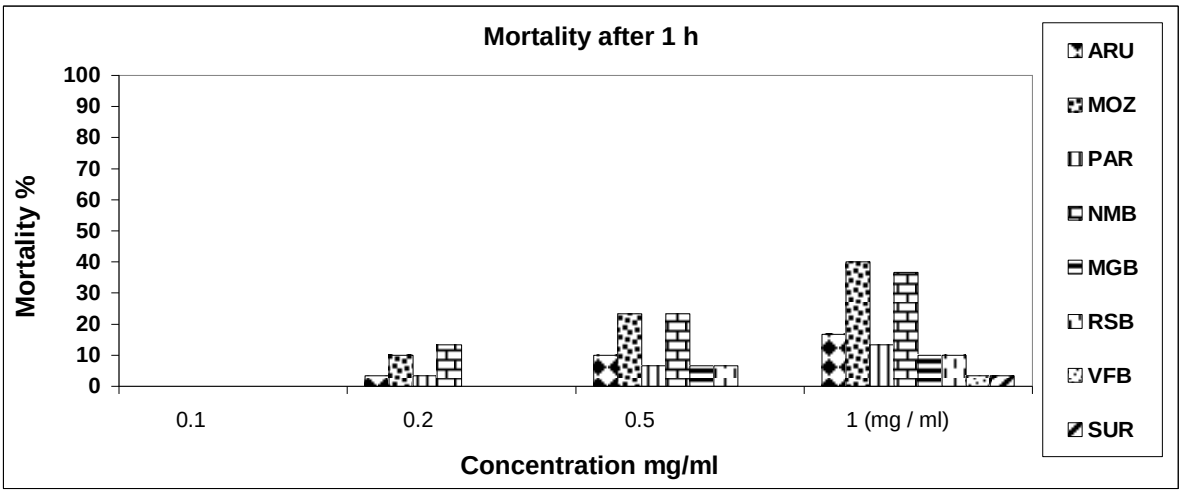


Figure 3. Mortality after 1 h of exposure of larvae to different concentrations (1 to 10%) of leaf aqueous extracts.

lowest toxicity because after 24 h and at a high concentration (10%), the mortality rate did not exceed 56% (Figure 5). Furthermore, the results of the larvicidal activity of leaves aqueous extract were subjected to

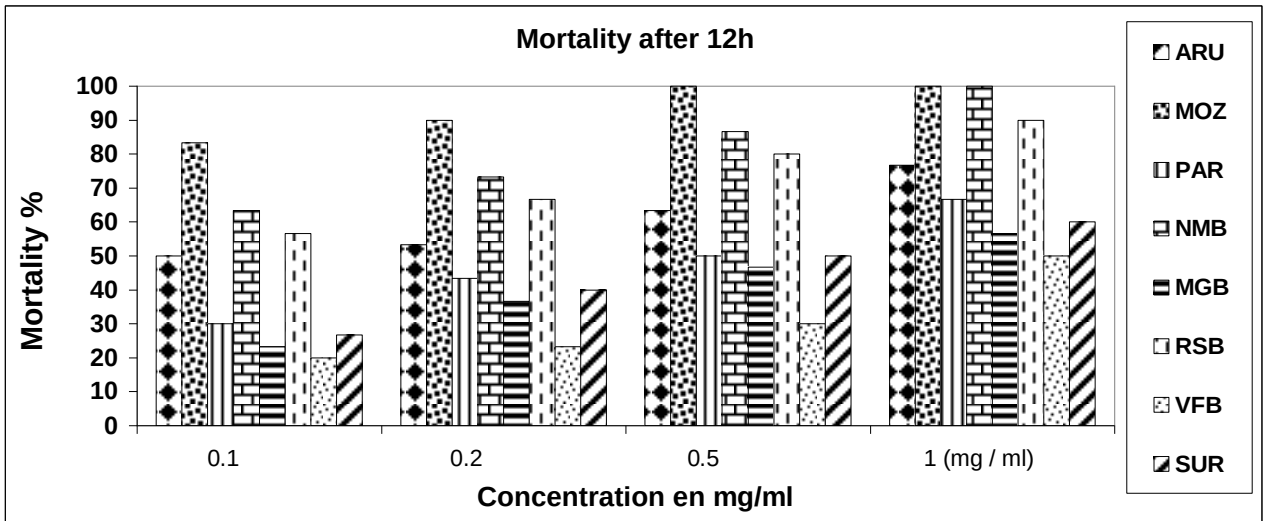


Figure 4. Mortality after 12 h of exposure of larvae to different concentrations (1 to 10%) of leaf aqueous extracts.

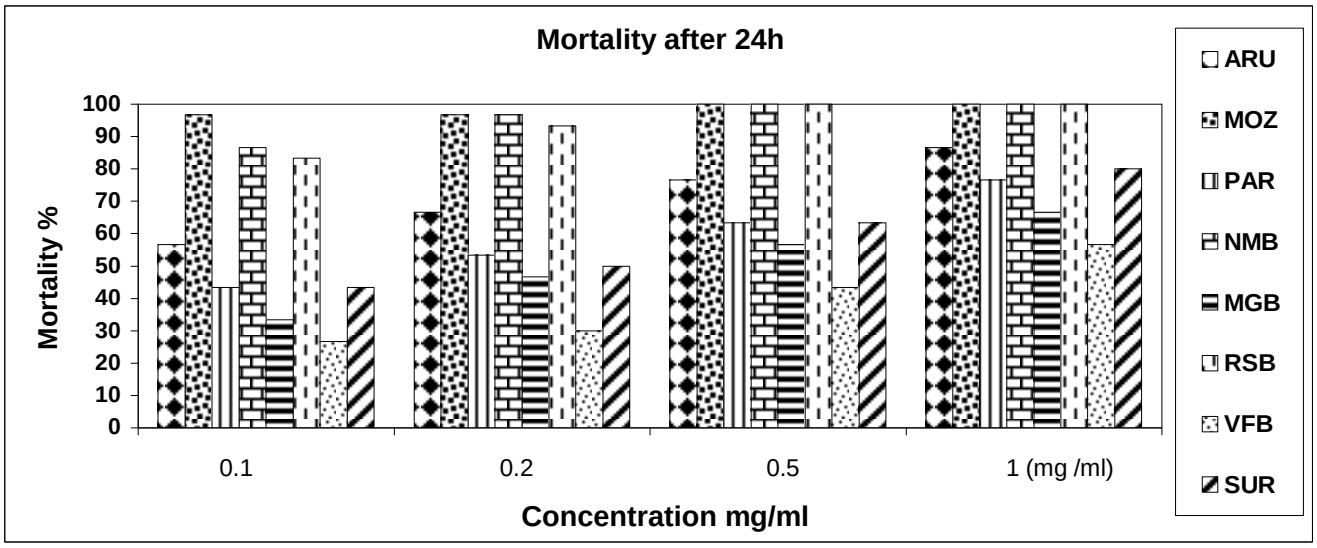


Figure 5. Mortality after 24 h of exposure of larvae to different concentrations (1 to 10%) of leaf aqueous extracts.

analysis of variance which highlighted significant differences of death percentage of the treated larvae between the provenances and among concentrations (Table 2). This reveals that the leaves aqueous extract is responsible for the susceptibility of *C. pipiens* larvae. In fact, Juliet et al. (2012) confirmed the presence of flavones like apigenin, orientin, vitexin, in *J. curcas* leaves. Hence, the efficacy of the leaf extract could be attributed to the presence of the flavones which can cause the toxicity to larvae.

It is noted that the percentage mortality increased with increasing concentrations of the leaves and seeds extracts. This effect is attributed to some well known toxic compounds such as alkaloids, steroids, flavonoids in *J. curcas* leaves extract, and oleic acid and linoleic acid in seeds extract of the same species, which are known to have insecticidal activities (Gutierrez et al., 2014; Elsayed Edriss et al., 2013; Khani et al., 2012). Moreover, the mortality of mosquito larvae was also increased in relation to the time exposure from 1 to 24 h (Figures 1 and 2) which confirmed what has been determined by several authors, who showed a progressive increase in mortalities in relation to time (Elsayed Edriss et al., 2013; Okeniyi et al., 2013; Shirvani Farsani et al., 2011; Adegbite and Adesiya, 2005). Indeed, the larvicidal activity of the highest concentration

Table 2. LC_{50} for seed and leaf aqueous extracts of eight provenances of *Jatropha curcas* L. against *Culex pipiens* L.

Provenance	Exposure period (h)	CL50 of seed aqueous extracts (%) ± SD	CL50 of leaf aqueous extracts (%) ± SD
ARU	24	0.52 ± 0.02	1.00 ± 0.06
MOZ	24	0.49 ± 0.01	0.50 ± 0.01
PAR	24	0.49 ± 0.04	1.41 ± 0.02
NMB	24	0.52 ± 0.04	0.50 ± 0.02
MGB	24	0.65 ± 0.06	2.90 ± 0.03
RSB	24	0.51 ± 0.02	0.50 ± 0.03
VFB	24	0.65 ± 0.02	4.72 ± 0.05
SUR	24	0.75 ± 0.05	1.00 ± 0.07

Mean of 3 repetitions; SD = Standard deviation.

(1 mg/ml) of the leaves and seeds extracts on *C. pipiens* L. larvae within 24 h of exposure increased the larvae mortality.

Lethal concentrations LC_{50}

The susceptibility of *C. pipiens* L. larvae to leaves and seeds extracts of *J. curcas* L. is illustrated in Table 2, showing the LC_{50} (Lethal concentration) values obtained after 24 h of treatment. The LC_{50} confirmed that seeds extracts are more effective than leaves extracts for most provenances. This potent activity is shown by lowest LC_{50}. Analyses showed that the seeds extract of Mozambique and Paraná (Brazil) provenances are the most effective against *C. pipiens* L. larvae and showed the lowest LC_{50} value (0.49%). However, the seeds extract of Surinam provenance showed the lowest toxicity against larvae. This low toxicity is confirmed by the highest LC_{50} value (0.75%). This toxicological study revealed that the leaves extract of Mozambique and Norte de Minas (Brazil) provenances were the most toxic with a LC_{50} (0.5%) while the leaves extract of Vale do Fequitinhonha (Brazil) provenance was the less toxic one (LC_{50} = 4.72%). These results are in agreement with other studies such as the one carried out by Tomass et al. (2011). This study affirms the larvicidal impact of crude methanol leaf extract of *J. curcas* and its column chromatographic fractions against the late third instar larvae of *Anopheles arabiensis*, the major vector of malaria in Ethiopia.

In addition, a study elaborated by Ojha and Pattabhiramaiah (2013), showed that the seed oil extract of *J.curcas* can be effectively used against *Aedes aegypti* and can be considered for eco-friendly vector control programs. Gutierrez et al. (2014) unveiled that larvicidal activity of *J. curcas* leaf is supported by the abundance of phytochemicals which show synergestic effects in terms of larvicidal action to mosquito larvae. Indeed, they determined the presence of alkaloids, steroids and flavonoids in *J. curcas* leaves. Alkaloids are known to possess medicinal and pesticidal properties. These compounds can be found in the whole *J. curcas* L. plant, but are more abundant in its seeds (Haas and Mittelbach, 2000). However, several studies have shown that the major factor responsible for *J. curcas* toxicity is the high concentration in the seeds of phorbolesters (tetracyclic diterpenoids) with known tumour promoting activity (Goel et al., 2007; Makkar et al., 1997). Other toxic compounds and anti-nutritional factors in the kernel and the seed cake include flavonoids, vitexine and isovitexine and 12-deoxyl-16-hydroxyphorbol (Aregheore et al., 2003). This larvicidal activity differ based on the plant species and the part used. The presence of several bioactive chemicals like alkaloids, steroids and flavonoids can be attributed to the susceptibility of the plant extracts as killing agent against mosquito larvae. The results reported here open the possibility for further investigation on the efficacy of larvicidal properties of natural product extracts.

Conflict of Interests

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

REFERENCES

Adegbite AA, Adesiya SO (2005). Root extracts of plants to control root-knot nematode on edible soybean. WJAS 1(1):18-21.

Aregheore EM, Becker K, Makkar HPS (2003). Detoxification of a toxic variety of *Jatropha curcas* using heat and chemical treatments, and preliminary nutritional evaluation with rats. S. Pac. J. Nat. Sci. 21:50-56.

Brunches J, Rhaim A, Geoffroy B, Angel G, Hervy JP (2000). Les *Culicidae* d'Afrique méditerranéenne. Logiciel de l'Institut de Recherche pour le Développement (I.R.D.), Montpellier, ISBN 2-7099-1446-8.

Chavan A, Gour VK, Basha H (2014). *Jatropha curcas* L: A predominant Panacea for Energy Security and Climate Change. Curr. World Environ. 9(1):130-136.

Dalziel JM (1955). The Useful Plants of West -Tropical Africa. Crown Agents for Oversea Governments and Administration, London.

Duke JA (1985). CRC Handbook of Medicinal Herbs. CRC Press, Boca Raton, FL.

Duke JA (1988). CRC Handbook of Medicinal Herbs. CRC Press, Boca Raton, FL.

Elsayed Edriss A, Satti AA, Alabjar ZA (2013). Larvicidal properties of two asclepiadaceous plant species against the mosquito *Anopheles*

arabiensis Patton (Diptera: *Culicidae*). J. Saudi Soc. Agric. Sci. 12 (1):59-66.
Goel G, Makkar HPS, Francis G, Becker K (2007). Phorbol esters: Structure, biological activity, and toxicity in animals. Int. J. Toxicol. 26:279-288.
Gutierrez PM, Antepuesto AN, Eugenio BAL, Santos MFL (2014). Larvicidal activity of selected plant extracts against the dengue vector *Aedes aegypti* Mosquito. Int. Res. J. Biol. Sci. 3(4):23-32.
Haas W, Mittelbach M (2000). Detoxification experiments with the seed oil from *Jatropha curcas* L. Ind. Crops Prod.12:111-118.
Hamilton MA, Russo RC, Thurston RV (1977). Trimmed Spearman Karber method for estimating median lethal concentration in toxicity bioassays. Environ. Sci. Technol. 11:714-719.
Heller J (1996). Physic Nut. *Jatropha curcas* L. Promoting the Conservation and Use of Underutilized and Neglected Crops. International Plant Genetic Resources Institute, Rome.
Juliet S, Ravindran R, Ramankutty SA, Gopalan AKK, Nair SN, Kavillimakkil AK, Bandyopadhyay A, Rawat AKS, Ghosh S (2012). *Jatropha curcas* (Linn) leaf extract - a possible alternative for population control of *Rhipicephalus* (*Boophilus*) *annulatus*. Asian Pac. J. Trop. Dis. 2(3):225-229.
Kaushik N, Kumar S (2004). *Jatropha curcas* L. Silviculture and Uses. Agrobios, India.
Khani M, Muhamad Awang R, Omarand D, Rahmani M (2012). Bioactivity Effect of *Piper nigrum* L. and *Jatropha curcas* L. Extracts against *Corcyra cephalonica* [Stainton]. Agrotechnology 2(1):1-6.
Luo MJ, Yang XY, Liu WX, XU Y, Huang P, Yan F, Chen F (2006). Expression, purification and anti-tumor activity of curcin. Acta Biochim. Biophys. Sin. 38:663-668.
Macedo J, Consoli RAGB, Grandi TSM, Dos Anjos AMG, de Olivira AB, Mendes NM, Queiroz RO, Zani CL (1997). Screening of *Asteraceae* (*Compositae*) plant extracts for larvicidal activity aginst *Aedes fluviatilis* (Diptera: *Culicidae*). Mem. Inst. Oswaldo Cruz. 92:565-570.
Makkar HPS, Becker K, Sporer F, Wink M (1997). Studies on nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. J. Agric. Food Chem. 45:3152-3157.
Mujumdar AM, Misar AV (2004). Anti-inflammatory activity of *Jatropha curcas* roots in mice and rats. J. Ethnopharmacol. 90:11-15.
Ojha K, Pattabhiramaiah. M (2013). Evaluation of Phytochemicals, Larvicidal Activity of *Jatropha Curcas* seed Ooil against *Aedes Aegypti*. Int. J. Appl. Res. Stud. 2 (12):1-12.
Okeniyi MO, Afolami SO, Fademi OA, Oduwaye OF (2013). Effect of botanical extracts on root-knot nematode (Meloidogyne incognita) infection and growth of cashew (*Anacardium occidentale*) seedlings. Acad. J. Biotechnol. 1(6):81-86.
Osoniyi O, Onajobi F (2003). Coagulant and anticoagulant activities in *Jatropha curcas* L latex. J. Ethnopharmacol. 89:101 - 105.
Rahuman AA, Gopalakrishnan G, Venkatesan P, Geetha K (2008). Larvicidal activity of some *Euphorbiaceae* plant extracts against *Aedes aegypti* and *Culex quinquefasciatus* Diptera: *Culicidae*. Parasitol. Res. 1025:867-873.
Shaalan EA, Canyon D, Younes MWF, Abdel-Wahab H, Mansour AH (2005). A review of botanical Phytochemicals with mosquitocidal potential. Environ. Int. 31:1149-1166.
Shirvani Farsani N, Zamani AA, Abbasi S, Kheradmand K (2011). Insecticidal Effects of Two Plant Aqueous Extracts against Second Instar Larvae of *Lycoriella Auripila* (Diptera: *Sciaridae*). WASET 5:10-23.
Silva GN, Faroni LRA, Sousa AH, Freitas RS (2012). Bioactivity of *Jatropha curcas* L. to insect pests of stored products. J. Stored Prod. Res. 48:111-113.
Solsoloy AD, Solsoloy TS (1997). Pesticidal efficacy of formulated *Jatropha curcas* L. oil on pests of selected field crops. In: G Gubitz, M Mittelbach, M Trabi (eds.) Proceedings from the symposium on Biofuels Industrial Products of *Jatropha curcas* L., Managua, Nicaragua. pp. 216-226.
Tewari JP, Shukla IK (1982). Inhibition of infectivity of two strains of water melon mosaic virus by latex of some angiosperms. GEOBIOS 9:124 - 126.
Tomass Z, Hadis M, Taye A, Mekonnen Y, Petros B (2011). Larvicidal effects of *Jatropha curcas* L. against *Anopheles arabiensis* (Diptera: *Culicidea*). MEJS 3 (1):52-64.

academicJournals

Vol. 13(26), pp. 2648-2660, 25 June, 2014
DOI: 10.5897/AJB12.2296
Article Number: 081C20445610
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic analysis of antibiotic production and other phenotypic traits from *Streptomyces* associated with seaweeds

Sridevi, K[1]* and Dhevendaran, K[2]

[1]Department of Aquatic Biology and Fisheries, University of Kerala, Kariavattom Campus, Thiruvananthapuram- 695 581, Kerala, India.
[2]School of chemical and Biotechnology, Sastra University, Thanjavur, Tamil Nadu 613401, India.

Received 4 July, 2012; Accepted 26 May, 2014

The Gram-positive bacterium such as streptomycetes known for its production of a diverse array of biotechnologically important secondary metabolites, have major application in health, nutrition and economics of our society. There are limited studies on the genetics of streptomycetes, especially seaweed associated *Streptomyces* sp. So, the present study made an attempt to study the genetics of production of antibiotic and other phenotypic properties was demonstrated by plasmid DNA curing analysis. The DNA-intercalating agent ethidium bromide was used to eliminate plasmid DNA from streptomycetes and effects of curing agent (EB) on the antibiotic production and loss of other phenotypic traits such as aerial and substrate mycelial production, biomass production, protein synthesis were studied. The study demonstrates that the ethidium bromide is potent and probably region-specific mutagens that are capable of inducing high rates of plasmid loss (curing), production of antibiotics was not eliminated, but was reduced by 20.2-79.8% and extracellular protein of 26 KDa mol.wt. was unaffected by curing agents. Data suggests that production of antibiotics and other phenotypic traits likely chromosomally encoded in marine *Streptomyces* species. The study concludes that the new methodologies such as mutasynthesis have contributed substantially to the discovery of additional antibiotics as an added feather to the scope of antibiotic industry.

Key words: Plasmids, genetics of *Streptomyces*, curing, phenotypic traits, antibiotic production.

INTRODUCTION

Microbial molecular genetics is gaining its momentum and popularity over the conventional methods in the classification of microbes. Genetic manipulation and conventional genetic analysis of actinomycetes (mainly streptomycetes) producing antibiotics and other secondary metabolites have high-lightened their possible

*Corresponding author. E-mail: kanaga_sridevi@yahoo.com, dheven.k@gmail.com.

involvement in the biosynthesis of plasmids, clusters of antibiotic biosynthesis gene and inter-specific gene-exchange. There are few studies regarding the genetic basis for the biosynthesis of these antibiotic compounds and other biologically active secondary metabolites. The genetic determinants for antibiotic compounds as well as other secondary metabolites in *Streptomyces* are carried on plasmids (Ishibashi, 1992; Kawachi et al., 2000).

Plasmids have been identified in many *Streptomyces* spp. and have been implicated in the control of a wide range of phenotypic properties, including the transfer of chromosomal markers ('fertility'), antibiotic biosynthesis and resistance, differentiation and melanin formation (Hopwood et al., 1986a; Chater and Kinashi, 2007) and production of secondary metabolites with variety of applications (Dharma raj and Dhevendaran, 2012). It has also been shown that various antibiotic-producing streptomycetes harbor plasmids, which vary in size and/or form depending on the antibiotic production (Hayakawa et al., 1979). The loss of antibiotic and other secondary metabolite production can occur upon treatment with "curing agents" (Coyne et al., 1984). However, with certain exceptions, genetic evidence for the existence of *Streptomyces* plasmids has been limited to studies on the effect of plasmid-curing agents on the stability of particular phenotypes, notably antibiotic production (Ismail et al., 1998; Hopwood, 2006). The biosynthetic genes for antibiotics in *Streptomyces,* which form a gene cluster, are usually located on chromosome (Keasling, 2008). In *Streptomyces coelicolor* A3 (2), the antibiotic biosynthetic genes for methylneomycin is located in extrachromosome (Marnix et al., 2010; Bentley et al., 2004). In some cases, the plasmid genes may encode 'regulatory function' while the structural genes are located on chromosomes (Ravel et al., 2000). The morphological differentiations that exist within their colonies, phenotypic characteristics and their genetic control mechanisms that regulate these events have been of great biological interest. The genetic determinants for the production of antibiotics and other phenotypic traits have not been extensively studied. Considering the above facts, the present study aimed at analyzing the effects of curing agent (EB) on the production of antibiotics, loss of other phenotypic traits and plasmid DNA from *Streptomyces* species. Therefore, the purpose of this study was to ascertain whether the genes for antibiotic and other phenotypic traits are either plasmid-borne or encoded on the chromosome in *Streptomyces* isolated from seaweeds.

MATERIALS AND METHODS

In the present study, seaweeds were collected from Muttom coast which is situated in the south coast of India located at a distance of 34 km from Cape Comorin at (8^{o} 7' 15" N: 77° 1'E) in India. Seaweeds were collected from the substratum along with the holdfast using a blunt knife and chiser. Seaweeds were transported to the lab in sterile polyethene bags immediately for further study.

Identification of seaweeds

The seaweed specimen was preserved in 4% formalin-seawater solution for further investigations. Some material was preserved in the form of herbarium sheets and kept in the Herbarium, Department of Botany, Holy Cross College. Cross sections (C. S.) of the material were obtained by free hands with the help of shaving blades, which were then stained with iodine, mounted in glycerine and sealed with the help of nail polish. Prepared slides were examined under microscope, and photographs were taken.

Isolation of *Streptomyces* from seaweeds

The seaweed extract were obtained by using the following method: 1 g mantle of live specimen was weighed, washed with distilled water and treated with 0.5% phenol for 5 min to inhibit the bacterial and fungal colonies present as contaminants. After washing with distilled water, the mantle was macerated. Aliquots of 1 ml of each seaweed extract was serially diluted with seawater to a dilution of 10^{-2}. A quantity of 1 ml of the dilutions were mixed with 20 ml of glycerol asparagine agar medium and incubated at room temperature (28 ± 2°C) for seven days. The *Streptomyces* colonies were counted and expressed as CFU/gm dry weight of visceral mass. The isolated strains were stored in ISP5 (glycerol asparagine agar Base) medium as agar slant cultures at 28 ± 2°C.

Characterization and identification of *Streptomyces* strain

Microscopic, cultural and physiological characteristics of *Streptomyces* strains were carried out by adapting the methods described by Shirling and Gottlieb (1966). Based on the characteristics, six strains were identified with the help of Nonomura keys (1974) and Actinobase database (Ugawa et al., 1989).

Isolation of plasmids from *Streptomyces*

Isolation of plasmid DNA from *Streptomyces* strains was carried out by alkaline lysis and potassium acetate precipitation. 2 ml of a 2- 4 day-old culture in FM medium were harvested by centrifugation (17,000× *g*, 4°C, 1 min). After washing with 1 ml of solution MP1 (Tris HCl-50 mM, EDTA-10 mM, RNase A 100 µg/ml), the cells were resuspended in 500 µl of solution MP1GL (Glucose-50 mM, Tris-HCl-25 mM, EDTA-10 mM, RNase A-100 µg/ml, Lysozyme-2-4 mg/ml) by vortexing. The suspension was incubated at 37°C for 30-60 min, then mixed with 500 µl of solution MP2 (NaOH-0.2 M, SDS-1%(w/v) by inversion and incubated at RT for 10 min. 400 µl solution MP3 (KAc.$3H_2O$-5 M) and 40 µl Rotiphenol® were added and mixed by inversion. The mixture was incubated on ice for 5 min. After 20 min centrifugation (20,000× **g**, 4°C), the supernatant was poured into a fresh microfuge tube and extracted twice with 300 µl Rotiphenol®. DNA was precipitated by addition of 0.8-fold volume of isopropanol and centrifugation (20,000× *g*, 4°C, 20 min). DNA pellet was washed with 500 µl 70% cooled ethanol, air dried and resuspended in 20-50 µl distilled water or TE buffer.

Agarose gel electrophoresis of DNA

Gel electrophoresis with 0.8-1% (w/v) agarose was used to separate DNA fragments. The buffer system employed was 1×TBE buffer. After running the gels, bands were detected under the UV light and photographed.

Plasmid DNA curing

Curing of plasmid was attempted by inoculating the suspension of

cultures into the Fermentation medium containing 10 mM EB. Broth cultures were incubated at 28°C in a shaker with 200 rpm up to sufficient growth. After sufficient growth, serial dilutions of cultures were made, each dilutions were plated onto GA agar medium and incubated at 28°C for 2-4 days. Presumptive aerial mycelium-negative colonies were picked up aseptically and re-placed onto same medium under the same conditions (modified protocol of Ismail et al., 1998)

Mycelial color determination

The cured and non-cured *Streptomyces* isolates were streaked on Petri dish containing glycerol asparagine agar, incubated at room temperature (28 ± 2°C) for seven days and mature sporulating aerial mycelium production was noticed. The cultures were classified into white, grey, red, green, blue, yellow and violet series depending on the aerial mycelial color. The color of the reverse side of *Streptomyces* colonies was observed; the strains were classified into pale yellow, olive or yellowish brown, cream depending on the substrate mycelial color.

Biomass production

The cured and non-cured isolates were cultured in broth medium under above said conditions. After sufficient growth, the cells were harvested by filtration. The wet minus dry weight of the filtrate was taken as growth.

Antimicrobial bioassay

Antibiotic activity of cured and non-cured *Streptomyces* strains was done against four different *Vibrio* spp. by disc diffusion method. Ten microlitre of *Streptomyces* culture was drawn in sterile discs and placed over the vibrio agar plates pre-seeded with V*ibrio* spp. The zone of inhibition was measured after 48 h in mm.

Drug resistance bioassay

The antibiotic sensitivity was tested against each cured and non-cured *Streptomyces* strain. Twelve antibiotic discs viz., gentamicin (10 µg), lincomycin (10 µg), penicillin-G (10 units), rifampicin (5 µg), streptomycin (10 µg), vancomycin (30 µg), amphotericin-B (100 units, 20 µg), chloramphenicol (30 µg), Erythromycin (15 µg), kanamycin (30 µg), nystatin (100 units), and tobramycin (10 µg) used in this piece of study were obtained from Hi-Media Pvt. Ltd., India. As per the specification, the concentration of each antibiotic was maintained. A suspension of the *Streptomyces* isolate was prepared to a particular McFarland standard (0.5 mcf), and then spread evenly onto a Muller-Hinton agar (M173) in a Petri dish. The commericial antibiotic disc was impregnated onto the medium and each plate was incubated at 28±2°C for 48 h during the study. After incubation, the occurrences and sizes of inhibition zones around the discs of the different antibiotics were tabulated (modified protocol of Rajput et al., 2012).

Extracellular protein profile

The strains of cured and non-cured isolates were inoculated onto GA broth medium, and incubated under submerged condition for four days. After four days, the twelve ml of culture was centrifuged at 1000 rpm for 10 min. The supernatant was collected and equal volume of 10% TCA (Tri Chloro acetic acid) solution was added. They were kept under refrigeration for over-night incubation (for precipitation). The precipitate was centrifuged at 1000 rpm for 15 min. Then, 50 or 100 µl of 1x PBS solution was added to the pellet and mixed well. This extracellular protein was stored at -20°C for further use (modified protocol of Subashkumar et al., 2007). Extracellular protein separation was made by 12.5% of SDS-gel SDS-PAGE electrophoresis (Lamelli, 1970).

RESULTS

Isolation and characterization of *Streptomyces* isolates

In the present study, 16 seaweeds were collected at monthly interval from Muttom coast, South India. The seaweeds were identified as *Gracillaria corticata, Chnoospora minima, Sargassum weightii, Spyridia hypnoides, Enteromorpha intestinalis and Hypnea valentiae, Gelidium microptera, Chatetomorpha media, Sargassum longifolium* etc., belonging to the order of Rhodophyta, Phaeophyta, Chlorophyta. For identification of Seaweeds, fixed in formalin, herbarium sheets were prepared and photographed and classified using guide (Dhargalkar, 2004). Seaweeds are the rich source of protein than cereals, egg and fish. Marine algae are not only the primary and major producers of organic matter in the sea, but they also exert profound effects on the density and distribution of other inhabitants of the marine environment. An understanding of the wide range of behavioral relationships that exist among organisms would provide us with clues to substances of biomedical interest. Secondary metabolites produced by the seaweeds and host organism to protect themselves and to maintain homeostasis in their environment (Sheeja, 1994; Dhevendaran et al., 2004; Kolanjinathan and Stella, 2011)

The maximum streptomycetes population (from 73-68 × 10^2) was observed in *Sargassum weightii*, *Enteromorpha intestinalis* and *Ulva lactuca*. The minimum *Streptomyces* population was (14- 5 × 10^2) observed in seaweeds such as *Chaetomorpha media* and *Hypnea valentiae* (Figure 1). Moreover, the streptomycetes strains were isolated using four different media such as Kusters agar, actinomyces agar, glycerol asparaginase agar and potato dextrose agar, and among the four media, GA medium obtained maximum number of streptomycetes isolates compared to other media (Figure 1). The present study is correlated with the findings of Anithakumary and Dhevendaran (2004) and Prasheetha (2008). Forty-five strains of *Streptomyces* different in aerial and substrate mycelial coloration were selected for the study. Among them, 15 isolates, which showed antimicrobial activity (Figure 2) against any one of four different *Vibrio* species (*V. harveyji*, *V.parahemolyticus*, *V. alginolyticus*, *V. vulnificus*) were tested for the presence of plasmids; out of them, six isolates having plasmids were further characterized by adopting the methods of International *Streptomyces* Project (ISP). The colonies of *Streptomyces* were slow-growing and had powdery

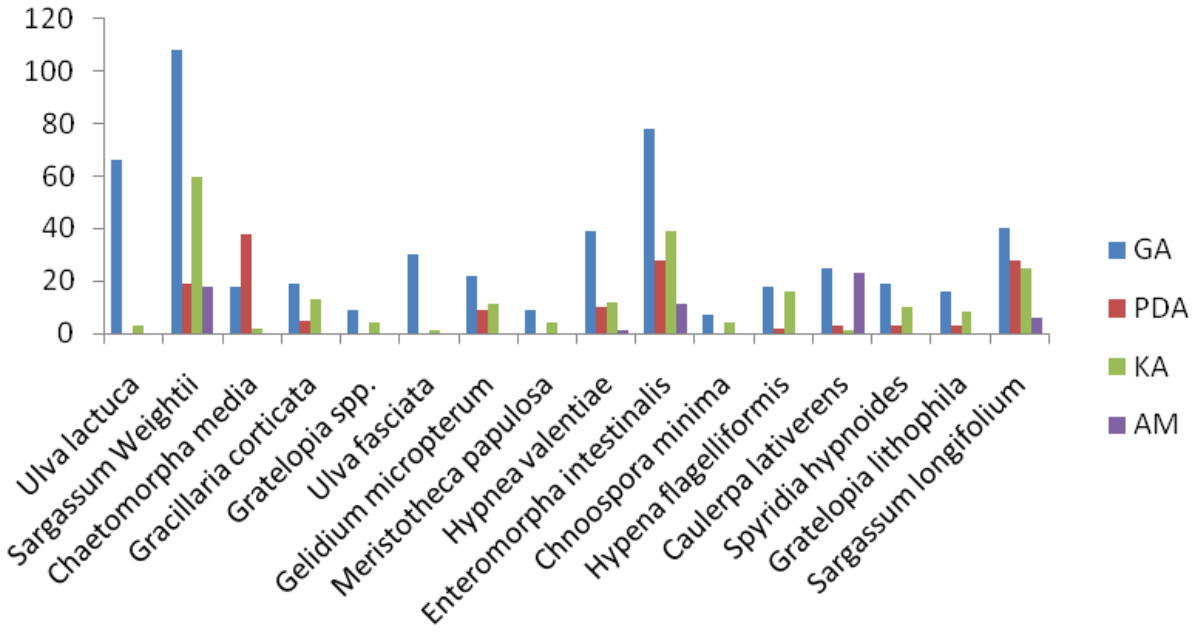


Figure 1. *Streptomyces* population recorded in different seaweeds in different months.

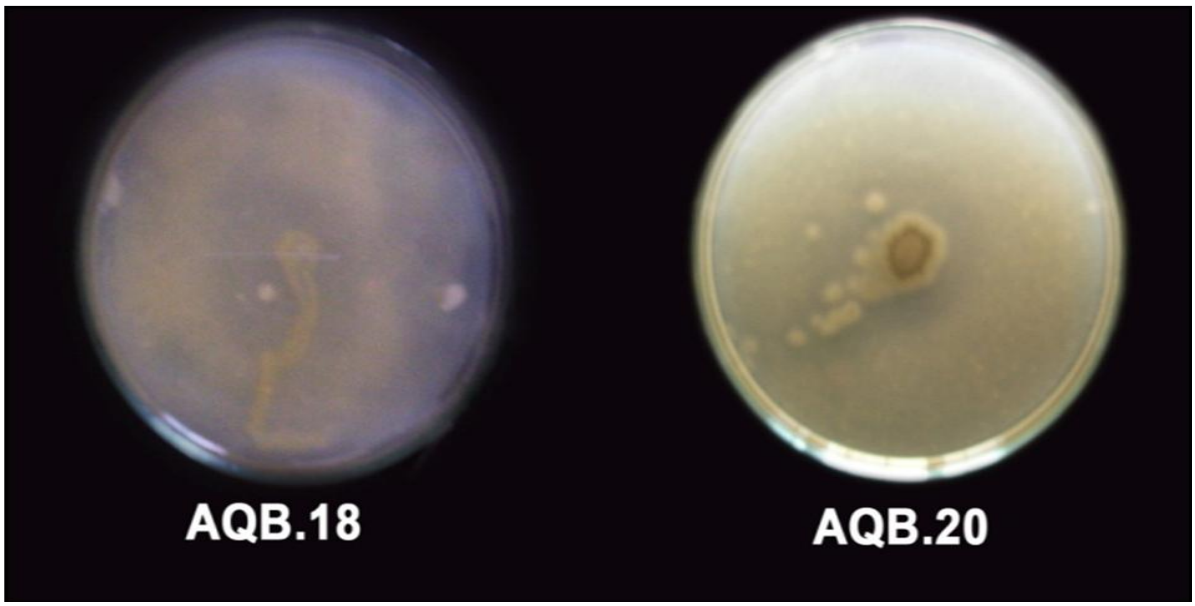


Figure 2. Antimicrobial activity of *Streptomyces* isolates against *Vibrio* sp. by double layer method.

appearance. Aerial mycelial colors of isolated strains were yellow, green, grey, white and bluish green and substrate mycelial color of each strain was very different like brown, reddish brown, red and yellow (Table 1). The different aerial and substrate mycelial color were due to the utilization of different carbon source for the growth (Vanajakumar, 1981). Most of the strains showed Rectiflexibles spore morphology; the strain AQB.SKKU8 showed hock smooth spore morphology (Figure 3) and among the six strains, only one strain produced melanoid and soluble pigments (Table 1).

Less than 10% of various sources of *Streptomyces* are known for melanoid production and grey colour series usually produce smooth spore surface compared to other (Mathew, 1995; Lakshmanaperumalsamy, 1978). The glucose, arabinose and xylose were well utilized and

Table 1. Phenotypic characteristics of *Streptomyces* spp. isolated from seaweeds.

Name of closely related species	Strain number	Seaweed source	Spore chain morphology	Spore surface	Aerial mass color	Reverse side color	Melanoid Pigment	Soluble Pigment	Carbon utilization								
									Glu	Ara	Xyl	Ino	Man	Fru	Rha	Suc	Raf
S. coelicolor	AQB.SKKU8	*Gracillaria corticata*	hock	Smooth	Yellow/ Green	Brown /lavender	-	-	+	+	+	-	+	+	±	±	-
S. autotrophicus	AQB.SKKU10	*Chnoospora minima*	RF	Smooth	Pale yellow	ND/ cream	-	-	+	+	+	+	+	+	+	+	-
S. pedanensis	AQB.SKKU18	*Sargassum weightii*	RF	Smooth	White	Brown	-	-	+	+	+	±	+	-	±	+	-
S. deccanensis	AQB.SKKU20	*Spyridia hypnoides*	SC	Hairy	Grey	Yellow	+	+	+	+	+	+	+	+	+	+	+
S. vinaceus	AQB.SKKU25	*Enteromorpha intestinalis*	RF	Smooth	Yellowish brown	Reddish brown	-	-	+	-	+	-	+	+	-	+	-
Streptomyces Nov sp.	AQB.SKKU37	*Hypnea valentiae*	RF	Smooth	Bluish green	Reddish Brown/red	-	-	+	±	+/	+	+/	+/	+	+	±

Abbreviations: RF,Rectiflexibles; SC, Straight chains; ND, not distinctive; Glc, D-glucose; Ara,L-arabinose, Fru, D-fructose; Ino, iinositol; Man, D-Mannitol; Raf, raffinose, Rha, L-rhamnose. Suc, sucrose; Xyl, D-xylose, "+": well utilized;" +/_": poorly utilized; "_": not utilized; S, *Streptomyces; Nov* sp.

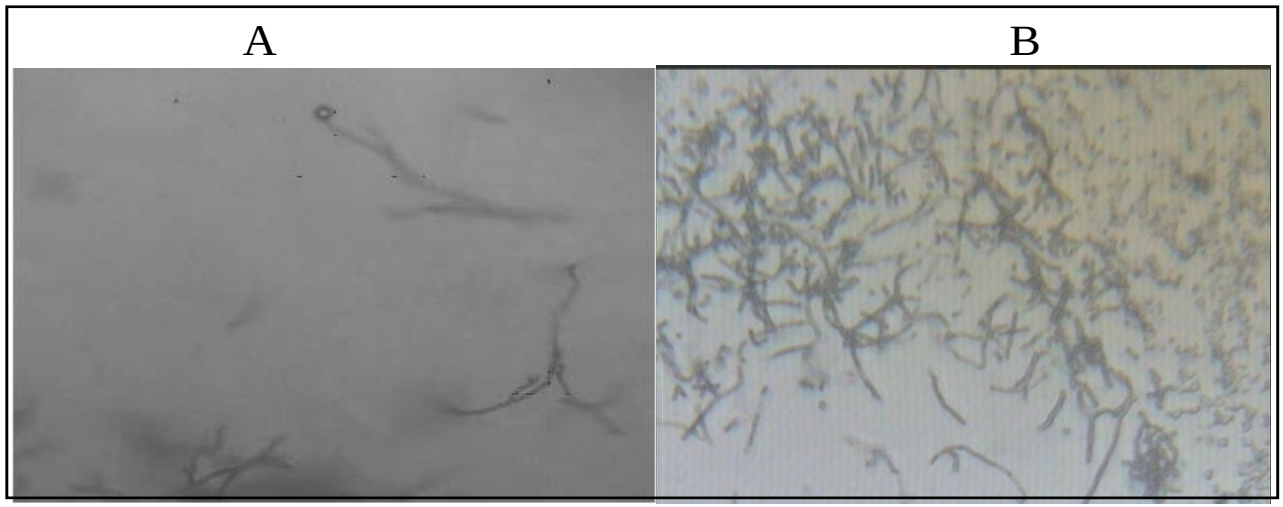


Figure 3. Gram-staining photograph smooth morphology of **A.** hock (strain AQB.SKKU 8) **B.** Rectiflexibile (strainAQB.SKKU18).

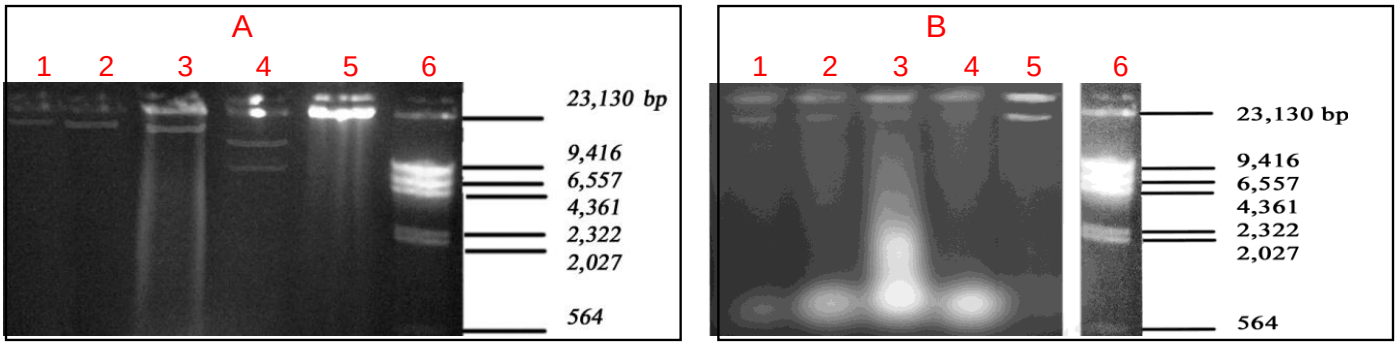


Figure 4. Plasmid DNA separated in Agarose gel electrophoresis**. (A)** Before curing; **(B)** After Curing; Lane 1, Strain AQB.SKKU8*;* Lane 2, MTCC 1540; Lane 3, Strain AQB.SKKU18; Lane 4, Strain AQB.SKKU25*;* Lane 5, Strain AQB.SKKU37; Lane 6, Marker (Hind III digest).

raffinose was not utilized by some strain (AQB.SKKU 8, 10, 18, 20); the rhamnose and sucrose was moderately utilized by the strain (AQB.SKKU 8, 18, 25). The strain AQB.SKKU 37 poorly utilized the entire carbon source. The *Streptomyces* isolates of marine seaweeds did not utilized the carbon source such as raffinose. Strepto-mycetes isolated from marine sponge *Mycale mytilorum* (Annandale), and *Tendania anhelans* (Lieberkuhn) showed abundant mycelium with glucose and xylose, moderate growth in medium containing arabinose, rhamnose, galactose, raffinose, mannitol and inositol, whereas growth was doubtful on media with fructose and sucrose (Selvakumar et al., 2010).

Plasmid profile before and after curing

Fifteen *Streptomyces* colonies associated with seaweeds were screened for plasmids. Of these, seven strains showed plasmids of varying sizes as their extra-chromosomal genetic material. Omura and coworkers (1981) from macrolide antibiotic producers conducted similar survey, and they found that only five of the 21 strains examined contained plasmids. The plasmid profile of *Streptomyces* species were resolved by agarose gel electrophoresis and it expressed multiple plasmids viz: major plasmids of 26and 23 kb and two minor plasmids of 17 and 8.8 kb (Figure 4A). Comparison of plasmid DNA profile of the six field strains of *Streptomyces* among themselves and with the reference strain (MTCC 1540) to catalogue the extent of homology indicated that, while strainAQB.SKKU.8 and strain AQB.SKKU18 carried a plasmids of 23 and 17 kb (Figure 4A) like that of reference strain. *Streptomyces* AQB.SKKU25 hosted multiple plasmids namely 26, 23.7, 14 and 8.8 kb. The strain AQB.SKKU10 showed the plasmids range from 0.8, 0.9 to 26 kb. The strain AQB.SKKU8, strain AQB.SKKU20 and strain *A*QB.SKKU18 showed unique plasmid patterns, which had homology with the reference strain *Streptomyces* MTCC 1540 before curing treatment (Figure 4A). Hayakawa et al. (1979) was found 17 kb linear plasmid in antibiotic resistance isolates of *Streptomyces*. The above results correlated with the previous findings in which the molecular weight 23.7 Kb plasmids were detected in antibiotic resistance *Streptomyces* species (Kinashi et al., 1987). The presence of multiple plasmids indicates the multiple antibiotic resistances and loss of plasmid after curing indicates the instability of plasmids treated with curing agents (Imran, 2009).

After curing treatment with EB, *Streptomyces* isolates and reference strain (MTCC 1540) lost the previously harbored plasmid. The strain AQB.SKKU8 and strain AQB.SKKU37 did not lose the high molecular weight plasmid (Figure 4B). The antibiotic production was also lost with high frequency from cultures treated with curing agents known to cause elimination of plasmids (Okanishi and Umezawa, 1978). In another report, plasmid-borne genes cured by dye treatment played a regulatory role (Akagawa et al., 1979).

Aerial and substrate mycelial coloration in wild and cured strains

The aerial and substrate mycelia production and coloration of *Streptomyces* strains before and after curing was attempted. The selected *Streptomyces* strains lost aerial mycelium after curing. The white color aerial mycelium was changed as pinkish white in AQB.SKKU18 and no color change was observed in strain AQB.SKKU25. The aerial mycelial color was different in each strain as shown in Figure 5A. The substrate mycelia color of *Streptomyces* strains were changed as pink, red and orange color after curing treatment (Figure 5B). The loss of aerial mycelium and different substrate mycelium production in *Streptomyces* may be due to the secondary metabolite production during germination of spores while treated with curing agents (Schaeffer, 1969). It has also been reported that the formation of genetically mapped

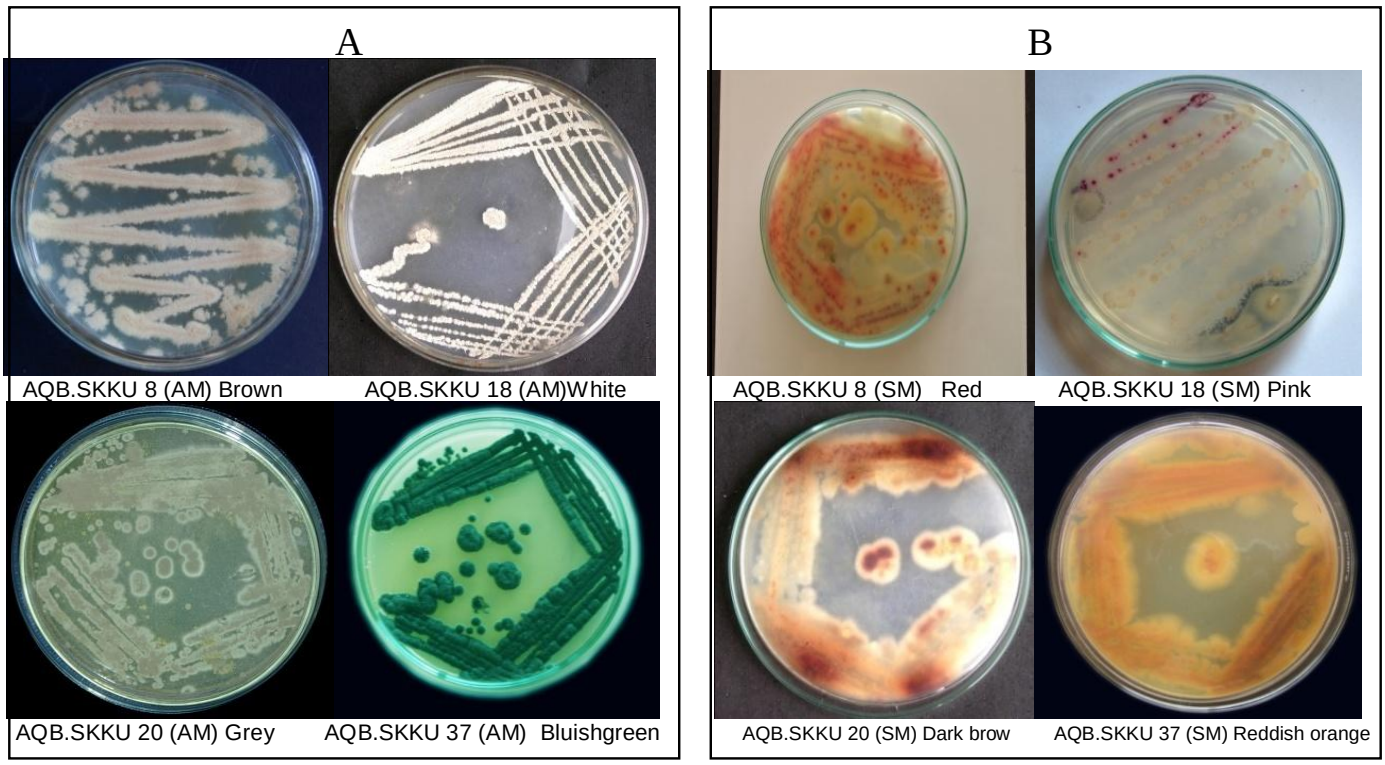


Figure 5. Different Mycelial coloration of *Streptomyces* isolates. **(A)** Before Curing; **(B)** After Curing; AM, Aerial mycelium; SM,Substrate mycelium.

mutants lacking in aerial mycelium is controlled by chromosomal genes rather than plasmids (Merrick, 1979; Imran, 2009).

Biomass production in cured and non-cured strains

The variation in biomass production was observed in all strains before and after curing treatment. The biomass production was decreased after curing and it was increased in strain AQB.SKKU25 (Figure 6). It indicated a re-directing of cell-energy toward primary metabolism (Ikeda et al., 1981; Ismail et al., 1998).

Antimicrobial bioassay before curing and after curing

Among the plasmid-isolated strains, experiments were performed in cured and non-cured *Streptomyces* isolates to find the presence or absence of antibiotic activity against three different *Vibrio* spp. Among the seven strains, all the *Streptomyces* isolates and reference strain MTCC 1540 showed similar antagonism against pathogenic *Vibrio* spp. In the case of strain AQB.SKKU20, the antagonistic activity was expressed towards *V. alginolyticus* only after curing treatment (Figure 7). The present also supported by Hopwood (1999), he described in a separate experiment, he picked seven *Streptomyces* strains that had failed to reveal any antimicrobial activity. After irradiating them with X-ray, five gave rise to antibiotic-producing variants. Thus, the study demonstrates that mutation stimulates antibiotic production in some cases (Flickinger et al., 1990; Imran, 2009; Kim et al., 2011).

Drug resistance bioassay in non-cured and cured isolates

The antibiotic resistance of six isolates and one reference strain MTCC 1540 (*S. griseus*) was tested before and after curing treatments. The resistance pattern of *Streptomyces coelicolor* AQB.SKKU8 and reference strain was similar. The isolated strains showed resistance to most of the tested antibiotics before curing. The strain AQB.SKKU18 and strain AQB.SKKU20 was resistant to all antibiotics used. Strain AQB.SKKU10 and strain AQB.SKKU37 were sensitive to the antibiotics such as Amphotericin-B and Nystatin before curing treatment. The strain AQB.SKKU25 showed sensitivity to Gentamycin, Streptomycin and Chloramphenicol before curing treatment (Table 2). The reference strains were homology with *Streptomyces* isolates in the resistance pattern of antibiotics such as Lincomycin, Penicillin-G

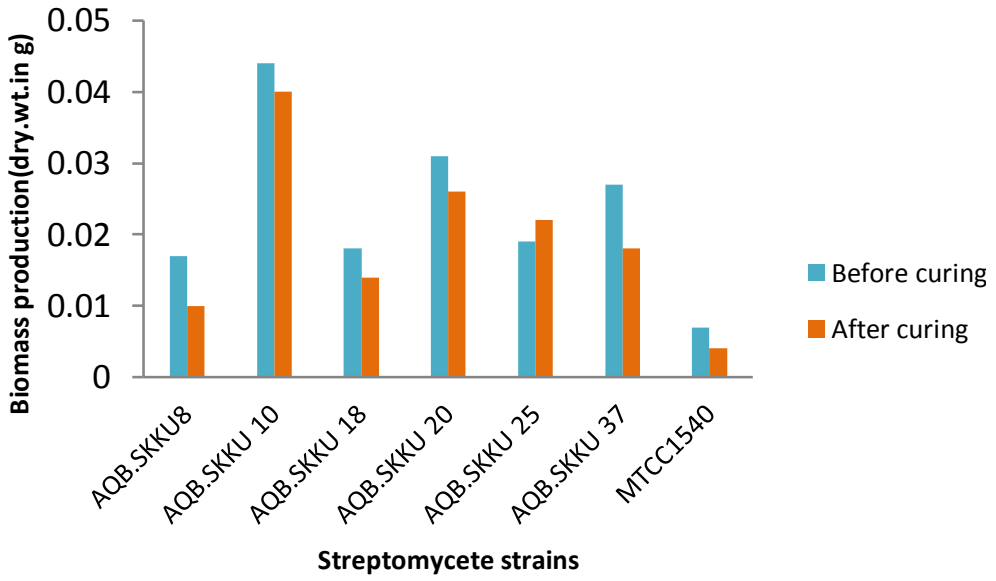


Figure 6. Biomass production of *Streptomyces* spp. before and after curing.

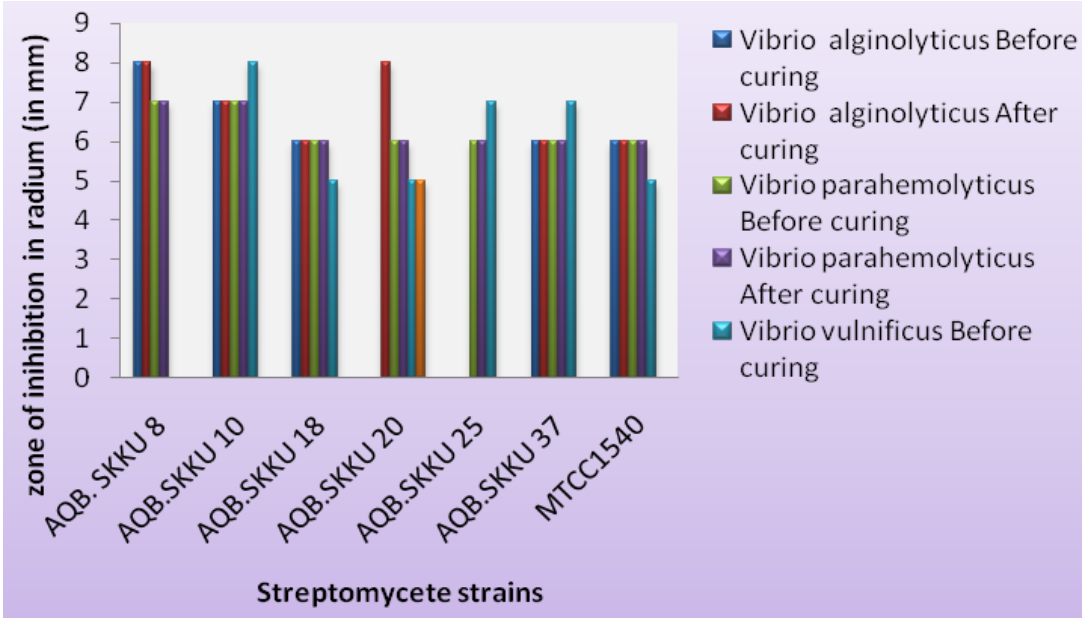


Figure 7. Antibacterial effect of *Streptomyces* sp. against pathogenic *Vibrio* spp. before and after curing.

and vancomycin. These are the resistance pattern of *Streptomyces* isolates and reference strain before curing. After curing, the resistance of *Streptomyces* species decreased compared to selected antibiotics before curing; it was reduced gradually after curing. The reference strain (MTCC1540) and the isolates of streptomycetes diameter of inhibition zone was reduced or increased and in some cases, sensitive strain become resistant and vice versa after curing which depends on the strain. The isolated *Streptomyces* also showed similar pattern (Table 2 and Figure 8B), which had homology with the plasmid profile also (Table 2). Characterization of *Streptomyces* strains in terms of their resistance to certain ranges of antibiotics makes it possible to avoid the repeated screening of isolates with the production of known antibiotics. It has been observed that *Streptomyces* species with mutagenic agents (also curing agents such as EB, AF and AO) cause strains to

Table 2. Antibiotic resistance of *Streptomyces* isolated from seaweeds.

Name of the antibiotics	Zone of Inhibition (mm)													
	Strain*SKKU8*		Strain*SKKU10*		Strain*SKKU18*		Strain*SKKU20*		Strain*SKKU25*		Strain*SKKU37*		*MTCC1540*	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II
Gentamicin	23	13	R	16	R	26	R	23	22	28	R	26	26	20
Lincomycin	R	R	R	R	R	R	R	R	R	R	R	21	R	R
Penicillin-G	R	R	R	R	R	R	R	R	R	R	R	25	R	R
Rifampicin	25	15	R	15	R	21	R	13	R	16	R	26	17	23
Streptomycin	16	R	R	15	R	27	R	22	11	20	R	R	28	34
Vancomycin	R	R	R	R	R	R	R	R	R	R	R	26	R	R
Amphotericin-B	R	R	11	22	R	R	R	R	R	R	22	20	R	R
Chloramphenicol	19	23	R	27	R	30	R	25	12	24	R	31	33	15
Erythromycin	R	15	R	23	R	R	R	14	R	16	R	30	28	R
Kanamycin	R	22	R	14	R	26	R	23	R	25	R	25	25	27
Nystatin	R	R	12	R	R	R	R	R	R	R	21	R	R	R
Tobramycin	R	14	R	21	R	16	R	20	R	12	R	12	R	15

I- Zone of inhibition before curing; II-Zone of inhibition after curing; R-resistant.

lose the ability to synthesize the secondary metabolites (Imran, 2009). Saadoun et al. (1998) suggested that antibiotic production is likely to be chromosomally encoded.

Extracellular protein expression in pre and post cured strains

The extracellular protein profiles of six strains of seaweed origins and one reference strain of MTCC 1540 (*S. griseus*) were studied. *Streptomyces* isolates exhibited below 26,000 and 43,000 Da as their extracellular protein. The similar ranges of proteins were also expressed in the reference strain (MTCC 1540) with SDS-PAGE separation. However, the expressed high molecular weight protein ranged between 43,000 to 66,000Da and above 66,000 to 97,4000Da and it differed in all pre-cured *Streptomyces* strains (Figure 9A). *Streptomyces* isolates and reference strain did not lose the molecular weight of 26,000Da protein during post-curing treatment (Figure 9B). The proteins present of *Streptomyces* strains in pre-curing was not expressed after curing, instead of that, new protein of high molecular weight (nearly 180,000 Da) was expressed in all *Streptomyces* isolate after curing (Figure 5B). Yang et al. (2012) reported the butanolide binding protein BarA of *S. virginiae* and has a molecular weight of 26,000 Da. In *S. griseus*, an A-factor binding protein had an apparent molecular weight of 26,000 Da (Miyake et al., 1989; Kawachi et al., 2000).

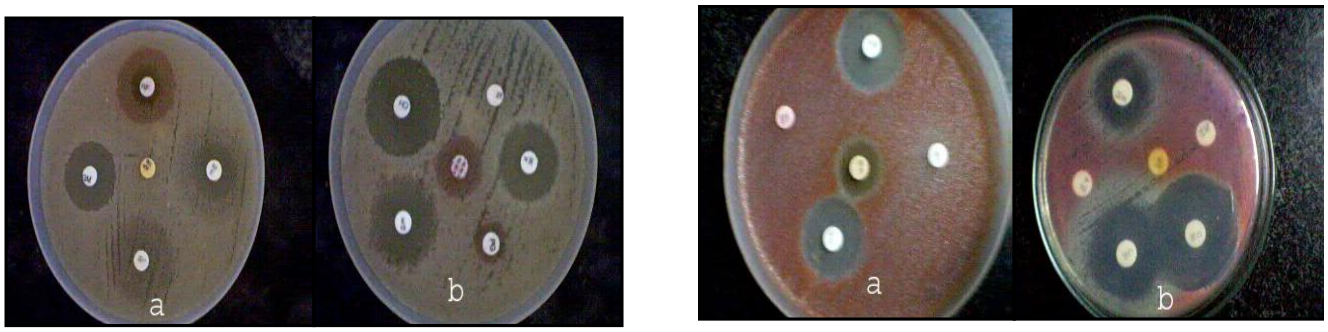
DISCUSSION

As secondary metabolites are frequently the result of complex, highly regulated biosynthetic process, a variety of changes in the genome may be necessary for the selection of high yielding derivatives of a wild strain. Baltz (2001) suggested that in many cases strain improvement have been achieved using natural methods of genetic recombination, which bring together genetic elements from two different genomes into one unit to form new genotypes; however, the most effective strategy is mutagenesis. A variety of chemical mutagens like ethidium bromide, N-methyl-N'-nitro-Nnitrosoguanidine (MNNG or NTG) are used for antibiotic and other secondary metabolite yield improvement in *Streptomyces* (Mamatha, 2009: Imran, 2009). These mutagens induce modifications of the base sequences of DNA that result in base pair substi-tutions, frame shift mutations, or large deletions that go unrepaired (Kieser et al., 2000). Little attention has made on the aspect of genetics study for the strain improvement and increase the yield of

Streptomyces MTCC 1540 *Streptomyces* AQB.SKKU 8

(A)

Streptomyces MTCC 1540 *Streptomyces* AQB.SKKU 8



Streptomyces AQB.SKKU10 *Streptomyces* AQB.SKKU20

(B)

Figure 8. (A) Antibiotic sensitivity of the selected *Streptomyces* isolates before curing. **(B)** Antibiotic sensitivity of the selected *Streptomyces* isolates after curing

biotechnology industry, especially *Streptomyces* from marine source other than soil such as seaweeds. Marine seaweeds have been in recent focus because they form close association with a wide variety of microbes and are rich source of biologically active secondary metabolites (Prasheetha, 2008). In this respect, *Streptomyces* strains were isolated from marine seaweeds and the genetic determinant for antibiotic production and phenotypic characteristics were analyzed by curing test. In the present study, *Streptomyces* species associated with marine seaweeds were identified with the help of Nonomura keys (1974) and Actinobase database

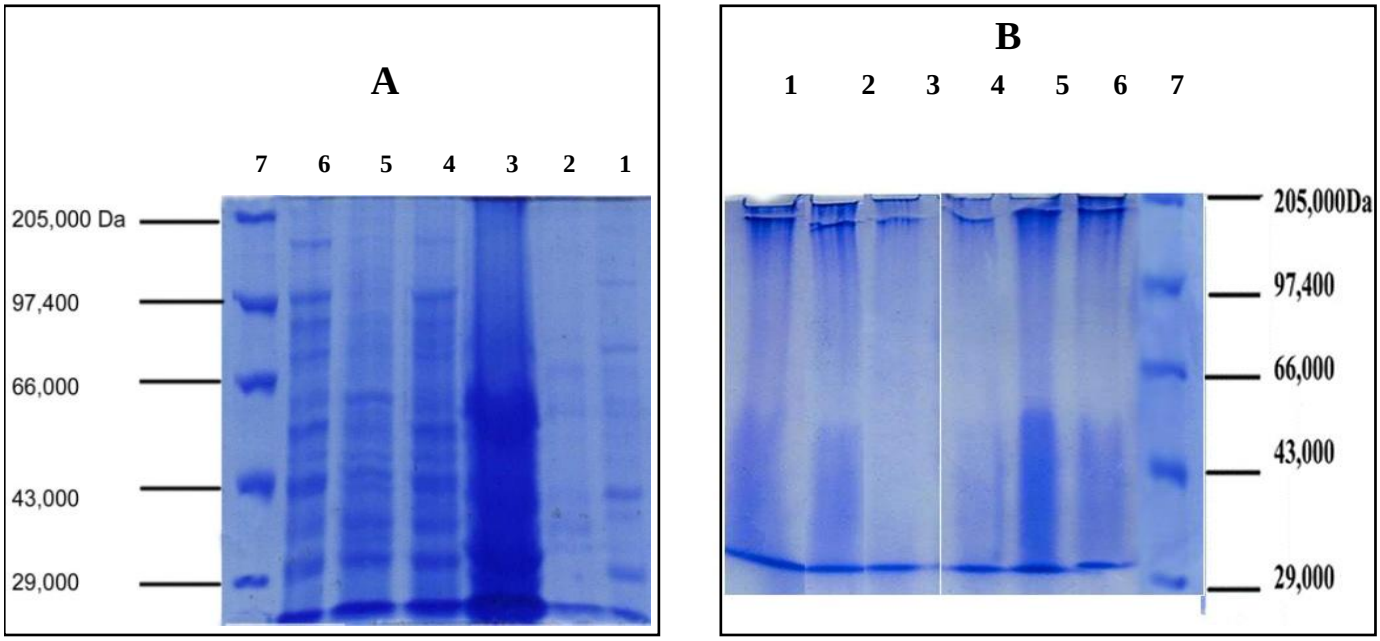


Figure 9. SDS-PAGE Separation of Extracellular protein from *Streptomyces* isolates. **(A)** Before curing; **(B)** After curing: Lane 1: Strain*SKKU8*; Lane 2, MTCC 1540; Lane 3, Strain*SKKU18*; Lane 4, Strain SKKU 20; Lane 5, Strain*SKKU37*; Lane 6, Strain*SKKU25;* Lane 7, Marker.

(Ugawa et al., 1989). Further molecular characterization is needed for taxonomic position. According to Shirling and Gottlieb (1966), unknown streptomycetes isolates can be characterized by the morphology, microscopic appearance, biochemistry, physiology, pattern of proteins and molecular genetics. It has also been reported that the exudates of seaweeds and slimes of the fish attracted microbes as the nutrient source and hambered them (Sheeja et al., 2011).

Detection of plasmid from various antibiotic producing strains strengthened the case for plasmid involvement in antibiotic production (Saadoun et al., 1998; Jorgensen et al., 2009). The study isolation and detection of plasmids were carried out in *Streptomyces* associated with seaweeds. After curing, *Streptomyces* isolates and reference strain (MTCC 1540) lost the previously harbored plasmid. Plasmidless variants had an altered secondary metabolism and a changed antibiotic resistance pattern (Akagawa et al., 1979). The *Streptomyces* colonies derived by treatment with EB appeared soft and did not form any characteristic aerial mycelium. The loss of plasmid after curing did not affect aerial mycelial production in all strains and it lost in some of the isolates indicates the genetic instability plasmid. It also indicates that aerial mycelium production is not plasmid mediated, but chromosomally mediated (Imran Sajid, 2009). The biomass production was decreased after curing. The studies on anticancer compound producing *Streptomyces* from marine algae reported decreasing biomass production after treatment with different concentration of pesticides (Prasheetha, 2008). The antagonism against *V. alginolyticus* was expressed only after curing in strain AQB.SKKU20; indicates the mutation stimulates the antibiotic production (Butler et al., 2002). Characterization of *Streptomyces* strains in terms of their resistance to certain ranges of antibiotics are used to avoid the repeated screening of isolates with the production of known antibiotics. In the present study, the streptomycetes isolates were resistant to antibiotics such as rifampicin and streptomycin. Earlier, antibiotic resistance of pesticide treated actinomycetes were studied, found rifampicin resistance actinomycetes from marine algae (Prasheetha, 2008). The diameter of inhibition reduced or increased after curing in some cases. The deduction is supported by Chater and Hopwood (1973). Volf and Altenbuchner (1998), supports the present study, who found genetic instability affect all the phenotypic properties and production of secondary metabolites and genes for primary metabolism.

An attempt on extracellular protein isolation of seaweed associated *Streptomyces* before and after curing was made in the present study. Previously harbored extracellular protein was lost after curing in *Streptomyces* sp. and expressed new protein. However, extracellular protein of 26000da (26KDa) was unaffected by curing treatment. It indicates that the 26KDa protein is genetically stable (Okamato et al., 1995). Butler (2003) also proved that extracellular molecules influenced antibiotic production in many streptomycetes. Furthermore, the experiments of plasmid curing with SDS

revealed that some catabolic genes were apparently plasmid-associated (AI Haixin, 2008). The present study on the effects of curing agents (Ethidium bromide) on the loss of linear plasmid DNA and further the generation of antibiotics in *Streptomyces* were revealed that the production of antibiotics was not eliminated, and isolates reduced by 20.2-79.8% in the plasmid cured strain. Similar survey conducted by Michaelson and Vinning (1978), who reported that exposure to Acriflavine and Ethidium bromide gave relatively large numbers of progeny that failed to produce the antibiotic or produced it in a much smaller quantities. Relationships between the genetic and phenotypic characteristics examined may provide preliminary insight into the distinct strategies that microbes use in optimizing their fitness in natural environments (Anita et al., 2006).

This is the first report on the genetic characterization of *Streptomyces* isolated from marine seaweeds based on the phenotypic characteristics. Multiple antibiotic resistance patterns can be regarded as useful marker phenotype for predicting the types of antibiotics and other metabolites which streptomycete produced. The data of the present study suggest that antibiotic and other phenotypic characteristics are chromosomally encoded in seaweed associated *Streptomyces* species. The study offers the new promises to modern molecular genetics world for the productivity and yield of secondary metabolites, which could be increased to many folds by selectively mutating the target genes for possible exploration of industrial strain for biotechnological application.

ACKNOWLEDGEMENTS

The authors wish to thank, Dr. Seetha Lekshmi (Life-Teck Research Centre), and Dr. Stella Mary, Department of Botany, M. S. University, India for their help in identification of seaweeds.

REFERENCES

Akagawa H, Okanishi M, Umezawa H (1979). Genetics and biochemical study of chloramphenicol non-producing mutants of *Streptomyces venezuelae* carrying plasmid. J. Antibiot. 32:610-620.

AI Haixin, ZHOU Jiti, Hong LV, WANG Jing, GUO Jianbo, LIU Guangfei, QU Yuanyuan (2008). Location and PCR analysis of catabolic genes in a novel *Streptomyces* sp.DUT AHX capable of degrading nitrobenzene. J. Environ. Sci. 20:865-870.

Anita Pandey, Pankaj Trivedi, Bhavesh kumat, Lok Man S Palani (2006). Characterization of a Phosphate Solubilizing and Antagonistic Strain of *Pseudomonas putida* (B0) Isolated from a Sub-Alpine Location in the Indian Central Himalaya. Current Microbiol. 53(3):102-107.

Anithakumary YK, Dhevendaran K (2004). Studies on *Streptomyces* associated with three selected seaweeds of kovalam coast. Kerala. Res. Utilin. 26:253-259.

Baltz RH (2001). Genetic methods and strategies for secondary metabolite yield improvement in actinomycetes. Antonie Van Leeuwenhoek. 79 (3-4): 251-9, PMID 11816967.

Bentley SD (2004). SCP1, a 356,023 bp linear plasmid adapted to the ecology and developmental biology of its host, *Streptomyces coelicolor* A3(2). Mol. Microbiol. 51:1615-1628.

Butler MJ (2003). Deletion of scbA enhances antibiotic production in *Streptomyces lividans*. Appl. Microbiol. Biotechnol. 61:512-516.

Butler MJ, Bruhein PM, Jovetic S, Maarinelli F, Postma P, Bibb MJ (2002). Engineering of primary carbon metabolism for improved antibiotic production in *Streptomyces lividans*. Appl. Env. Microbiol. 68:4731-4739.

Chater KF, Hopwood DA (1973). Differentiation in acionmycetes. In Microbial Differentiation eds. Ashworth JM and Smith JE, Cambridge,Cambridge University Press. pp.143-160.

Chater KF, Kinashi H (2007). *Streptomyces* linear plasmids: their discovery, functions, interactions with other replicons, and evolutionary significance. Microbiol. Monogr. 7:1.

Claus Jorgensen, Andrew Sherman, Ginny IC, Andrian Pasculescu, Alexie Poliakov, Marylin Hsiung, Brett Larsen, David GW, Rune Linding, Tony Pawson (2009). Cell-specific Information processing in segregating populations of Eph Receptor- ephrin-expressing cells. Science. 11;326(5959):1502-1509.

Coyne VE, Usdin K, Kirby R (1984). The effect of inhibitors of DNA repairs on the genetic instability of *Streptomyces* Cattleya. J. Gen .Microbiol. 130:887-892.

Dhargalkar VK (2004). Seaweeds- a field manual. pp.14-24.

Dhevendaran K, Sukumaran M, Georgekutty MI (2004). Growth hormone (IAA) producing bacteria and streptomycetes and their biofertilizer effect on mangrove seedling. *Avicennia officialis*. L. Mar. Biotechnol. 6:209-213.

Flickinger MC, Greenstein M, Bremmon C, Conlin J (1990). Strain selection, medium development and scale-up of toyocamycin production by streptomyces chrestomyceticus. Bioprocess Engineering, 13(4):143-153.

Hayakawa T, Tanaka T, Sakaguchi K, Otake N, Onehara HY (1979). A linear plasmid-like DNA in *Streptomyces* spp producing lankacidin group antibiotics. J. Gen. Appl. Microbiol. 25:255-260.

Hopwood DA (1999). Mutation induce Antibiotiic productiovity.*Streptomyces* in nature and medicine, the antibiotic markers books. p.187.

Hopwood DA (2006). Soil to genomics: the *Streptomyces* chromosome. Annu. Rev. Genet. 40:1-23.

Hopwood DA, Kiesser T, Lydiate DJ, Bibb MJ (1986a). *Streptomyces* plasmids. Their Biology and uses as cloning vector. In: Antibiotic-producing *Streptomyces*. The Bacteria, Vol.IX (S.W.Queener and E.Day, eds.), Academic Press, New York. pp.159-299.

Ikeda H, Tanaka H, Omura S (1981). Genetic and biochemical feautures of spiromycin biosynthesis in *Streptomyces ambofaciens* RM 265.J6. J. Antibiot. 35:507-516.

Imran Sajid (2009). Screening for antibiotics from indigenous Streptomycetes, their genetic and mutational analysis. Ph.D thesis. Department of Microbiology and Molecular genetics University of Punjab, Quaid-e-Azam, Campus, Lahore.

Ishibashi Y (1992). Genetic studies into musty odor production by actinomycetes. Water Sci. Tech. 25:171-176.

Ismail saadoun, Ahmed Elbethiea, Willard T Blevins (1998). Mutagenesis by ethidium bromide and N-methyl-N'-nitro-N-nitrosoguanidine in oro-flavour compound producing strains of *Streptomyces*. J. Biosc. 23(5**)**:595-600.

Kawachi R, Akashi T, Kamitani Y, Sy A, Wangchaisoonthorn U, Nihira T, Yamada Y (2000). Identification of an AfsA homologye (BarX) from *Streptomyces virginiae* as a pleiotropic regulator controlling autoregulator biosynthesis, virginiamycin biosynthesis and virginiamycin MI resistance. Mol. Microbiol. 36:302-313.

Keasling JD (2008). Synthetic biology for synthetic chemistry. ACS Chem. Biol. 3:64-76.

Kieser T, Bibb M, Buttner J, Chater KF, Hopwood DA (2000). General Introduction to Actinomycetes Biology. In:practical *Streptomyces* genetics. The John Innes Foundation.

Kinashi H, Shimaji M, Sakai M (1987). Giant linear plasmids in *Streptomyces* which codes for antibiotic biosynthesis genes. Nature. 328:454-456.

Kolanjinathan K, Stella D (2011). Pharmacological Effect of *Gracilaria corticata* Solvent Extracts against Human Pathogenic Bacteria and Fungi. Inter. J. Pharmaceut. Biol. Arch. 2(6):1722-1728.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London). 227:680-685.

Lakshmanaperumalsamy P (1978). Studies on actinomycetes with special reference to antagonistic streptomycetes from sediments of Porto Novo coastal zone. Ph.D. Thesis, Annamalai University.

Mamatha SS (2009). Polyunsaturated Fatty acids of Mucor sp. with special reference to Gamma linolenic acid. Ph.D thesis. Universtiy of Mysore.

Marnix HM, Axel T, Andriy K, Marco van den Berg, Ulrike M, Wilbert H, Liang W, Mohammad TA, Catherine MR, William CN, Roel ALB, Rainer B, Eriko T (2010). The Sequence of a 1.8-Mb Bacterial Linear Plasmid Reveals a Rich Evolutionary Reservoir of Secondary Metabolic Pathways. Genome Biol. Evol. 2:212-224.

Mathew A (1995). Studies on antagonistic *Streptomyces* sp. associated with fish and shell fish of Veli Lake, Kerala. Indian. J. Exp. Biol. 12:32-54.

Merrick MJ (1976). A Morphological and Genetic Mapping Study of Bald Colony Mutants of *Streptomyces coelicolor.* J. Gen. Microbiol. 96:299-3I5.

Michaelson AM, Vinning LC (1978). Loss of chloramphenicol production in strains of *Streptomyces* species 3022a treated with acriflavine and ethidium bromide. Can. J. Microbiol. 24:662-669.

Miyake K, Horinouchi S, Yoshida M, Chiba N, Mori K, Nogawa N, Morikawa A, Beppu T (1989). Detection and properties of A-factor binding protein from *Streptomyce griseus* J. Bacteriol. 1171:4928-4302.

Nonomura H (1974). Key for classification and identification of 458 species of the *Streptomyces* included in the ISPP. J. Ferment. Technol. 52:78-92.

Okamoto S, Nakamura K, Nihira T, Yamada T (1995). Virginiae butanolide binding protein from *Streptomyces virginiae*. Evidence that Vbr A is not the Virginiae butanolide binding protein and reidentification of the true binding protein. J. Biol. Chem. 270:12319-12326.

Okanishi M, Umezawa H (1978). Plamids involved in antibiotic production in *Streptomyces*, In E. Freerksen, I.Tarnok, and J.H. Thumin (ed.), Genetics of the actinomycetales.Gustav Fischer Verlag, Stuttgart. pp. 19-38.

Omura S, Ikeda H, Tanaka H (1981). Extraction and characterization of plasmids from macrolide antibioticproducing streptomycetes. J. Antibiot. 34:478-481.

Prasheetha PK (2008). Studies on anticancer and antibiotic compounds from streptomycetes associated with seaweeds of Cape Comorin. Ph.D Thesis, Kerala University.

Ravel J, Wellington EM, Hill RT (2000). Interspecific transfer of *Streptomyces* giant linear plasmids in sterile amended soil microcosms. Appl. Environ. Microbiol. 66:529-534.

Saadoun I, Mohammad MJ, Al-Momani F, Meqdam M (1998). Diversity of soil streptomycetes in Northern Jordan. Actinomycetes. 9:52-60.

Schaeffer P (1969). Sproulation and the production of antibiotics, enzymes and exotins. Bacteriol. Rev. 33:48.

Selvakumar Dharmaraj, Ashokkumar B, Dhevendaran K (2010). Isolation of marine *Streptomyces* and the evaluation of its bioactive potential. Afr. J. Microbiol. Res. 4(4):240-248.

Selvakumar Dharmaraj, Dhevendaran K (2012). Evaluation of Streptomyces as a probiotic feed for the growth of the ornamental fish Xiphophorus helleri. Food Technolol. 48(4):497-504.

Seon-Hye Kim, Han-Na Lee, Hye-Jin Kim, Eung-Soo Kim (2011). Transcriptome Analysis of an Antibiotic Downregulator Mutant and Synergistic Actinorhodin Stimulation via Disruption of a Precursor Flux Regulator in *Streptomyces coelicolor*. Appl. Environ. Microbiol. 77(5): 1872-1877.

Sheeja K (1994). Bacteria and antagonistic *Streptomyces* sp. associated with seaweeds of Kovalam coast. M. Phil, Thesis, Kerala University.

Sheeja MS, Selvakumar D, Dhevendaran K (2011). Antagonistic Potential of Streptomyces Associated with the Gut of Marine Ornamental Fishes Middle-East. J. Sci. Res. 7(3):327-334.

Shirling EB, Gottlieb D (1966). Co-operative descriptive of type cultures of *Streptomyces*. The International *Streptomyces* Project. Intr. J. System. Bacteriol. 17:315-322.

Subashkumar R, Vivekanandhan G, Suresh SSR, Natarajaseenivasan K, Thayumanavan T, Lakshmanaperumalsamy P (2007). Typing of *Aeromonas hydrophila* of fish and human diarrhoeal origin by outer membrane proteins and lipopolysaccharides. Indian J. Microbiol. 47:46-50.

Ugawa Y, Sugawa K, Kudo K, Sugawara H, Tateno Y, Seino Y (1989). Actinobase: An image database for identification of Actinomycetes. Trends in Actinomycetology (Japan), pp. 17-20.

Vanajakumar S (1981). Studies on actinomycetes associated with mollusks from Porto Novo coastal zone. Ph.D. Thesis, Annamalai University.

Volf JN, Altenbuchner J (1998). Genetic instability of the *Streptomyces* chromosome. Mol. Microbiol. 27:239-246.

Yogita Rajput, Jayant Biswas, Vibhuti Rai (2012). Potentiality Test in Antimicrobial Activity and Antibiotic Sensitivity of Subterranean *Streptomyces* strains Isolated from Kotumsar Cave of India. Intr. J. Biol. Chemis. 6(2):53-60.

Yung-Hun Yang, Eunjung Song, Ji-Nu Kim, Bo Rahm Lee, En-Jung kim, Sung-Kee Park, Woo-Scong Kim, Hyung Yeon Park, Jong-Min Jeon, Thangamani Rajesh, Yun-Gon Kim, Byung Kee Gim (2012). Characterization of new Scb R-like γ-butyrolactone binding regulator (SIbR) in *Streptomyces coelicolor*. Appl. Microbiol. Bioteechnol. 96(1):113-121.

academicJournals

Vol. 13(26), pp. 2661-2667, 25 June, 2014
DOI: 10.5897/AJB2014.13860
Article Number: 9A8C6A645611
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Effects of wall materials and lyophilization on the viability of *Weissella confusa*

Liliana Serna-Cock*, Vladimir E. Vallejo-Castillo and Estefanía Garcia-Gonzalez

Facultad de Ingeniería y Administración, Universidad Nacional de Colombia Sede Palmira, Cr 32 No. 12-00 Chapinero, Vía Candelaria, Palmira - Valle del Cauca - Colombia.

Received 15 April, 2014; Accepted 16 June, 2014

The effects of wall materials and encapsulation by lyophilization on the viability of *Weissella confusa* were evaluated. Aloe vera gel, sodium casein at 5 and 15% p/v, sodium alginate at 2% p/v, buffer phosphate, and a mixture (Aloe vera gel, sodium casein, and sodium alginate) as wall materials, were used. Bacteria without encapsulation (*W. confusa*) as control were used. Encapsulated bacteria were freeze dried for 48 h, in order to determine their viability in the freezing and sublimation-drying stages. Results indicate that bacteria without encapsulation, showed greater loss of viability in the sublimation-drying stage. All the wall materials evaluated, may be used for encapsulation of bacteria, because, at the end of the freeze-drying process, the encapsulated bacteria showed higher viability percentages than non-encapsulated bacteria, with significant statistical difference ($p<0.05$). The protective effect of wall materials was higher in the sublimation-drying stage, compared to freezing stage.

Key word: Aloe, *Weissella*, probiotic, encapsulation.

INTRODUCTION

Probiotic lactic acid bacteria (LAB) are useful for the dairy and nutraceutical industry, due to their applications to human and animal health (Reddy et al., 2009). Several studies have demonstrated the probiotic potential of *Weissella confusa*, its antimicrobial activity against pathogenic microorganisms, including *Helicobacter pylori* (Nam et al., 2002), *Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli, Klebsiella pneumonic* (Serna-Cock et al., 2012), and its ability to adhere itself to the vaginal and intestinal epitheliums (Ayeni et al., 2011; Lee et al., 2012). In the food probiotics, processing is important; the concentration of probiotic bacteria (WHO / FAO, 2006), and the techniques are used to maintain their viability (Carvalho et al., 2004).

Encapsulation techniques have been developed and successfully used in the preservation and protection of probiotic LAB. In encapsulation, the material used to trap the substance or microorganism to be encapsulated is called encapsulation material, cover membrane, shell, vehicle, wall material, or external phase matrix (Serna-Cock and Vallejo-Castillo, 2013). Encapsulation of LAB reduces damage caused by external factors such as storage conditions (time, temperature, moisture, oxygen)

*Corresponding author. E-mail: lserna@unal.edu.co. Tel: + 57 + 2 + 286 88 88. Fax: + 57 + 2 + 286 88 88.

(Burgain et al., 2011), and also, the encapsulation of LAB, decreases the degradation of bacteria in the human gastrointestinal tract, where the pH is less than 2.00 (Kailasapathy, 2006). Lyophilization (freeze drying), is an encapsulation technique consisting in the elimination of water of a product, by means of sublimation of free water in the solid stage, (previous freezing of the product), followed by vacuum pressure application (Abdelwahed et al., 2006).

Sublimation occurs when vapor pressure and ice surface temperature are below water triple point (Song et al., 2005). Lyophilization is one of the best methods to conserve the properties of biological products (Shui et al., 2006). Lyiophilization-encapsulated probiotics are more stable along storing, especially at low temperatures and inert atmospheres (nitrogen or vacuum) (Manojlović et al., 2010).

Khoramnia et al. (2011) used skimmed milk, sucrose, and lactose as wall materials in the lyophilization-encapsulation of *Lactobacillus reuteri*; these authors report viabilities of 96.4 and 73.8%, for 6-month storage at 4 and 30°C, respectively. Carvalho et al. (2003) evaluated the effect of sorbitol and monosodic glutamate in a skimmed milk solution at 11% on the viability of stored *Lactobacillus bulgaricus, Lactobacillus plantarum, Lactobacillus rhamnosus, Enterococcus durans,* and *Enterococcus faecalis,* using encapsulation by lyophilization.

The findings show that sorbitol and monosodic glutamate maintain the viability of the strains along prolonged storage, with no significant differences between the viability of encapsulated and free cells. Chan et al. (2011), obtained cell viabilities of 5%, using liofilization and sodium alginate (2%) and sodium caseinate (10%) as wall materials, for stabilizing the viability of encapsulated cells. *Lactobacillus paracasei ssp. paracasei F19 (Lactobacillus F19)* and *Bifidobacterium lactis Bb12* in sodium caseinate (15% w/w) were microencapsulated, and retained the cell viability in 16 and 43%, respectively (Heidebach et al. (2010). Sodium caseinate offer suitable physical and functional properties for microencapsulation, due to its amphiphilic character and emulsifying characteristics (Hogan et al., 2001). Studies show improving viability when different types of wall materials as polysaccharides and proteins were included. Hence, cell viability during the lyophization encapsulation process is affected by the type of strains, the parameters of the lyophilization process, the physiological cell state, and the use of cryoprotectors (Abadias et al., 2001). Thus, is necessary to carry out specific encapsulation studies for each type of strain.

The main causes leading to cell viability loss during lyophilization are, ice formation and high osmolarity, (resulting from high internal solutes concentration) which causes cell membrane damage, macromolecular denaturelization, and water loss (Huang et al., 2006). The choice of a cryoprotector is important to maintaining the viability of LAB during the dehydration and storage stages (Carvalho et al., 2004). The most commonly used cryoprotectors include skimmed milk, glycerol, manitol, sorbitol, trealhose, sucrose, maltose, fructose, and proteins (Abadias et al., 2001; Carvalho et al., 2004; Gbassi et al., 2009; Huang et al., 2006). However, the most important selection criterion of an encapsulation material is its functionality in respect to the probiotic (Nedovic et al., 2011). At present, there is a high interest in Aloe vera for the food industry, thus being used as functional nutrient in drinks and ice cream (Martínez-Romero et al., 2006). In spite of its functionality, Aloe vera has not been used as wall material in encapsulation.

A mixture between carbohydrates and proteins can improve the effectiveness of encapsulated probiotics. Therefore, the aim of this present study was to evaluate the effects of wall materials and the lyophilization on the viability of *W. confusa*. The wall materials were Aloe vera gel, sodium casein at 5 and 15% p/v, sodium alginate at 2% p/v, buffer phosphate pH 7.5, and a mixture of pure Aloe vera, casein at 10% and alginate at 2% p/v. During the lyophilization process, the freezing and sublimation-drying stages were evaluated.

MATERIALS AND METHODS

Microorganism culture conditions

A cryoconserved strain, biochemically identified as *Weissella confuse*, isolated by Serna et al. (2010) was used. *W. confusa* strain, was replicated for three generations using MRS commercial substrate (De Man et al., 1960; Scharlau, Spain) (24 h at 37 ± 0.2ºC). For its growing, batch fermentation was used, following the methodology of Serna et al. (2010). After fermentation, *W. confuse* was separated from its metabolites, using centrifugation for 30 min at 5000 rpm (Eppendor Centrifuge 5804R, Germany). The bacteria were washed using 1 mL of NaCl at 0.9% and then centrifuged for 5 min at 5000 rpm. Finally, the supernatant was discarded (Picot and Lacroix, 2004).

Wall materials (encapsulation materials)

Sodium casein of 92.7% (alanate 180, Fonterra, New Zealandd), Sodium alginate (Sigma-Aldrich Co. USA), buffer phosphate, and Aloe vera (Aloe barbadensis Miller), were used as wall materials. In addition, a mixture of these materials was used. The phosphate buffer was selected, because this compound is used for the release of encapsulated cells. Aloe vera was obtained from an experimental plantation at Universidad Nacional de Colombia-Palmira, located 1,100 m above sea level.

Sodium casein was used in aqueous solutions at 5% p/v (C5) and 15% p/v (C15). The solutions were shaken for 12 h (Heidebach et al., 2010). Sodium alginate was used at 2% p/v (AG) (Kailasapathy, 2006). The buffer phosphate (BP) solution was used at pH 7.5. In order to produce Aloe vera gel, acibar (a yellow color liquid) was extracted by cutting the base of the leaf and leaving it drain for 1 h (Miranda et al., 2010). Then, the crystals from leaf epidermis were isolated and processed in a juice extractor (Black & Decker JE2200B, USA), under aseptic conditions. The frozen Aloe

Table 1. Viability percentage of *Weissella confusa* in different wall materials, during freezing and sublimation-drying.

Time (h)	AG	A	BP	C5	C15	FC	MZ
0	100.00 ± 0.00^{a}						
12	41.93 ± 2.03^{Ab}	97.57 ± 1.98^{Da}	92.71 ± 2.38^{Da}	90.33 ± 1.03C^{Dab}	90.14 ± 4.47^{CDa}	79.84 ± 0.12^{Bb}	83.11 ± 1.39^{BCb}
24	41.52 ± 2.46^{Ab}	96.58 ± 1.93^{Da}	91.00 ± 4.20^{CDa}	82.79 ± 6.47^{BCb}	88.65 ± 2.90^{CDa}	71.77 ± 0.16^{Bc}	81.85 ± 0.81^{BCb}
36	41.18 ± 1.24^{Ab}	88.10 ± 0.58^{Bb}	47.20 ± 4.57^{Ab}	80.67 ± 2.77^{Bb}	85.91 ± 6.39^{Ba}	45.16 ± 0.31^{Ad}	79.74 ± 0.84^{Bb}
48	40.87 ± 1.23^{CDb}	81.70 ± 0.50^{Fc}	46.77 ± 4.57^{Db}	36.51 ± 1.29^{BCc}	31.37 ± 0.43^{Bb}	0.02 ± 0.00^{Ae}	69.19 ± 0.96^{Ec}

Mean ± SD. FC = bacteria without encapsulation, C5 sodium casein at 5% p/v, C15 = casein at 15% p/v, AG = sodium alginate, A = Aloe vera, BP = buffer phosphate, MZ = mixture of Aloe vera, sodium casein at 10% and sodium alginate at 2% p/v.

vera gel (A) was stored at 5°C for 12 h and used undiluted. Additionally, a mixture (MZ) of Aloe vera, sodium casein at 10%, and sodium alginate at 2% p/v. was used (this mixture was evaluated in previous experiments).

Encapsulation process by freeze-drying

Bacteria without encapsulation (FC) in concentration of 10,344 ± 0,038 Log_{10}UFC g^{-1} were separately mixed with the corresponding wall materials, using a 1:4 ratio between the bacteria and the wall material (Brinques and Ayub, 2011). Encapsulated and non-encapsulated bacteria were freeze-dried (frozen at - 20°C, vacuum pressure 0.120 mbar and condensing temperature -50°C) using (Labconco, England). During the freezing process, cell count was made at 0, 12 and 24 h, and during the sublimation-drying process, cell count was made at 24, 26 and 48 h. The cell count made at 24 h in freezing process, corresponded to initial conditions of the sublimation-drying stage. FC treatment corresponded to treatment control. FC was freezing-dried to the same conditions described above (Doherty et al., 2010; Kailasapathy, 2006) (Doherty et al., 2010; Kailasapathy, 2006).

Quantification of living bacteria

For liberation of bacteria, the encapsulated bacteria were dissolved (1:10, v/v) in buffer phosphate (pH 7.5), and were centrifuged for 2 min at 5000 rpm (Eppendorf Centrifuge-5804R, Germany). Quantification of cell viability was done using spread-plate (agar MRS, 48 h, and 37 ± 0, 2°C). Afterward, plates containing 30-300 colonies were enumerated, expressing the counting in $UFCg^{-1}$ (Doherty et al., 2010, 2011).

Viability of the probiotic strain

Viability was assessed after freezing and sublimation-drying, in accordance with Doherty et al. (2010), using equation 1:

$$\% \text{ Viability} = (100 \times N/N_o) \quad (1)$$

Where N, is the number of viable cells after freezing in UFC g^{-1} and N_o, number of viable cells before freezing in en UFC g^{-1}.

During the sublimation-drying process, the percentage of viable cells was calculated using Equation 2:

$$\% \text{ Viability} = (100 \times N_t^*/N_o^*) \quad (2)$$

Where, N_t^* is the number of viable encapsulated cells in UFC g^{-1}, each time, along sublimation-drying and N_o^* is the number of viable encapsulated cells before freezing in $UFCg^{-1}$ (Semyonov et al., 2010).

Statistical analysis

A univariate design with 7 levels, FC, C5, C15, AG, A, BP and MZ was used. The response variable was the percentage of viability. Response variable was evaluated during the freezing and drying-sublimation processes, at the time t0 = 0 h, t1= 12 h, t2 = 24 h, t3 = 36 h and t4 =48 h. Results were presented as mean ± standard deviation (SD) of three replicates. The results were analyzed using SPSS 15.0 for Windows (SPSS Inc, Chicago IL, USA). The comparison between averages was made using Tukey, with a probability of $p< 0.05$.

RESULTS AND DISCUSSION

Table 1 shows the means of percentages of viability of *W.confusa* in different wall materials, during freezing and sublimation-drying times.

Different letters in the superscripts in the same column or row indicate significant differences, according to Tukey's comparison ($p <0.05$). Capital letters indicate significant differences ($p <0.05$) between different treatments (row). Lowercase letters indicate significant differences ($p <0.05$) for the same treatment during the time evaluated (column).

Percent viability of FC, presented statistically significant decrease during the freezing and sublimation-drying (Table 1). At the end of the freezing process (24 h), the viability of FC decreased to 28.23%, however its viability percentage was statistically equal to treatments C5 and MZ (Table 1). This indicates that C5 and MZ have no effect cell cryoprotectant in the freezing step. At the end of the process of sublimation-drying (48 h), FC had the lowest percentage of viability compared to all treatments, with statistical significance of $p<0.05$ (Table 1).

Figure 1 shows that after 36 h of process, the cell concentration, measured as $Log_{10}CFUg^{-1}$, decreased significantly reaching 6,722 ± 0.033 $Log_{10}CFUg^{-1}$ after 48 h (In Figure 1, axis Y was divided for including information of FC treatment). For all treatments, the behavior of the cell concentration during the sublimation drying process was similar to the behavior of % viability, since this percentage was calculated from the cell concentration.

This may be caused by cell stress produced by the

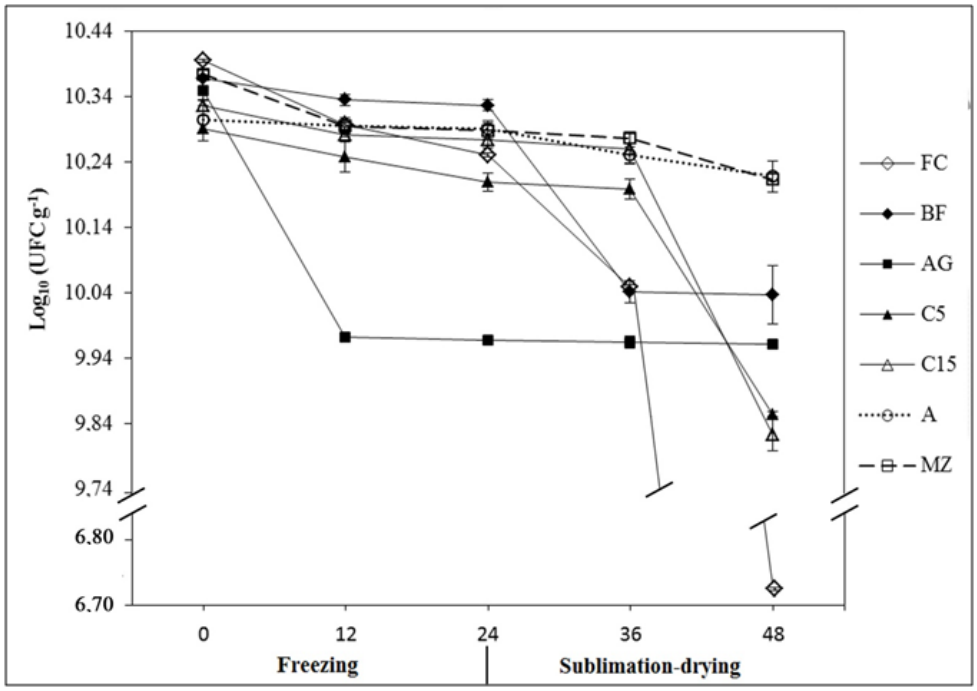


Figure 1. Cell count of *Weissella confusa* for different encapsulation materials during the freezing-sublimation stage of lyophilization. FC = bacteria without encapsulation; C5, sodium casein at 5% p/v; C15 = casein at 15% p/v; AG = sodium alginate; A = Aloe vera; BP = buffer phosphate; MZ = mixture of Aloe vera, sodium casein at 10% and sodium alginate at 2 % p/v.

formation of ice crystals during freezing and during the sublimation-drying stage in lyophilization (Otero et al., 2007). When bacteria are lyophilized, some membrane regions may be negatively affected, mainly because of water crystals in the freezing stage, as well as changes in membrane permeability and protein denaturalization (De Giulio et al., 2005; Yang et al., 2012).

At the end of sublimation-drying process (Table 1), the encapsulated bacteria with wall material A, showed the highest percentage of viability (81.70 ± 0.50%) ($p < 0.05$), however, from 36 h of processing, there showed a significant decrease in the viability percentage ($p < 0.05$), which shows that there is an adverse effect on the stage of sublimation-drying (Table 1). Referring to the cell concentration, at the end of the sublimation-drying process, 10.217±0.019 $\log_{10}$CFUg^{-1} was obtained, this being the highest value for all treatments. At the end of the freezing process, the encapsulated bacteria with the wall material A, showed no statistically significant differences with BP and C15 treatments (Table 1). Kanmani et al. (2011), used glucose and galactose at a 35% concentration as protecting substances for the probiotic *Enterococcus faecium* MC13, obtaining viabilities of 85.6 and 84.7%, after encapsulation by lyophilization, respectively. Zayed and Roos (2004) also evaluated a mixture of sucrose and trehalose for lyophilization encapsulation of *Lactobacillus salivarius*, obtaining 80% viability of the strain. The viability percentage obtained with Aloe vera gel as cryoprotector, is due to its high content of polysaccharides, such as mannose, glucose, and galactose (Chang et al., 2011). The use of sugar-based cryoprotectors (mono and disaccharides) produces high rates of viability (Chávez and Ledeboer, 2007; Huang et al., 2006; Khoramnia et al., 2011; Semyonov et al., 2010). This is attributed to the protection that exerted the sugars on the functionality of cell proteins. The sugars create a glassy matrix during the lyophilization stage, which presents high viscosity and low mobility. Additionally, the increase in cell viability is attributed to the fixation of solutes to cell proteins, due to the fact that the solutes behave as a substitute for water, when the zones of protein hydration are altered as a result of drying (Carvalho et al., 2004). Aloe vera has high glass transition temperature (Tg=70°C in lyophilization) (Nindo et al., 2010), and this gives additional protection to the cells, compared to other polysaccharides such as sucrose. The drying temperature in this study was 25°C, when a biological product is stored below the glass transition temperature, the chemical reactions such as oxidation of free radicals is slowed and the cellular damage is limited (Fu and Chen, 2011). The main bioactive compound of Aloe vera is acemannan, a water-soluble carbohydrate, and consists of glucose and manose monomers linked by glucosíde β–(1,4) bonds

(Femenia et al., 2003; Reynolds and Dweck, 1999). Aloe vera pulp contains 93% in dry base of polysaccharide, of which 62.9% is mannose, 13.1% is glucose and 1.5% is galactose (Ni et al., 2004). Carvalho et al. (2004), evaluated the influence of the addition of different sugars on the survival of *L. bulgaricus,* during freeze-drying, the study showed that the presence of mannose produced higher rate of survival of the bacteria, after freeze-drying. Abadias et al. (2001), evaluated during freeze-drying, the viability of *Candida sake*, when it was coated with different protective materials. They obtained viabilities of 0.2% when concentration of glucose and fructose of 1% were used, and viabilities of 1% when galactose solution to 1% was used. The acemannan plays an important role in the healing of wounds, due to inhibiting bacterial growth and promoting macrophage activity (Djeraba and Quere, 2000), therefore, Aloe vera provides functional advantages, compared with other wall materials.

The MZ treatment viability decreased, in the first 12 h of processing and at the end of the sublimation-drying process. The viability showed statistically different values, compared to the other treatments. These findings are in accordance with those reported by Nanasombat and Sriwong (2007), they used mixtures of skimmed milk, lactose, sucrose, and trehalosa in different combinations, for the lyophilization-encapsulation of *Lactococcus lactis* 13IS3 and Lactobacillus sakei 13IS4, and they obtained viabilities of 61 and 75% for *L. lactis* and 64 and 74% for *Lactobacillus sakei*. The viability percentages of a mixture of materials were used and higher compared with not mixed materials.

Collagen, trehalosa, L-cisteín and glycerol in the encapsulation by lyophilization of Bifidobacterium longum BIOMA 5920, were used. Viabilities of 83% using mixtures of wall materials were obtained, and viabilities of 53.22% using no-mixture wall materials were obtained (Yang et al., 2012). Gbassi et al. (2009), used lyophilization, and sodium alginate (20 g/L) in combination with whey protein (10 g/L), to encapsulate *L. plantarum* 299v, *L. plantarum* 800 and *L. plantarum* CIP A159 strains. The researchers concluded that the combination of polysaccharides and proteins is a feasible alternative, since it improves cell viability. Furthermore, they assessed the viability of the strains in gastric and intestinal simulated juices; the results showed that encapsulated strains had higher viability in gastric juices than no-encapsulated cells. In intestinal juice only encapsulated bacteria maintained viability.

In buffer treatment, viability did not differ significantly with AG treatment. The buffer is used to release the cells from the capsules, and subsequently obtaining a cell count (Doherty et al., 2010, 2011). AG treatment, presented at 12 h of processing, the largest decrease in the percentage of viability. Values were statistically different compared to the other treatments (Table 1). After 12 h of the process, the percentage viability was statistically unchanged until the end of sublimation-drying process. This behavior was similar to that found in Chan et al. (2011); they indicated that the sodium alginate do not protects cells during lyophilization, due to physical properties of sodium alginate cause cellular stress.

Between all encapsulation materials, C5 and C15 treatments had the lowest percentage of viability at the end of sublimation-drying process (cell concentration of 9.854 ± 0.003 and 9.823 ± 0.003 $Log_{10}CFUg^{-1}$, respectively) (Figure 1). In these two treatments after 36 h, the greatest decrease in the percentage of viability was observed. C5 treatment did not differ significantly with treatments AG and C15 to 48 h of processing. These findings are similar to those reported by Heidebach et al. (2010); they used lyophilization with sodium casein at 15% to encapsulate *Bifidobacterium* Bb12 and *Lactobacillus* F19, obtaining viabilities of 40 and 30%, respectively.

The low viability percentages found in this study can be attributed to the ability of bacteria to survive in different ways under the same adverse or comfort conditions (Carvalho et al., 2004; Meng et al., 2008). Therefore, for each strain there should be an evaluation of different encapsulation materials in order to find the most convenient strain (Carvalho et al., 2003; Otero et al., 2007). Further research is necessary to test new wall materials and determine other variables such as viscosity, molecular weight, gelification, composition, *Tg*, and other properties that can be useful in technical applications and materials optimization, in order to enhance viability.

Conclusions

Using wall materials, the life of the bacteria was protected in higher percentage in the sublimation-drying stage. Bacteria without-encapsulation showed higher viability decrease in the sublimation-drying stage (24-48 h).

All wall materials evaluated in this study, have potential in the encapsulation of lactic acid bacteria, due to, cell counts at the end of the process, are found within the ranges accepted by several conuntries, for probiotics foods (at least 7 to 9 Log 10 probiotic cultures per serving of product). Thus, *W. confusa* encapsulated can be used in the formulation of probiotics.

Aloe vera gel is a promising material for the encapsulation of active compounds because it improves the functionality of the material to be encapsulated. In this study, Aloe vera was the only one wall material that maintained the viability of *W. confusa* above 80%. Likewise, buffer phosphate was found to be a cheap material that could be used as a complement in the formulation of wall materials.

Encapsulation of *W. confusa* expands the application horizons of this lactic acid bacteria to the food industry, including foods with probiotic effects, as application in

milk fermented, desserts, ice cream, and powdered starter culture (for fermentation process).

Conflict of Interests

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

REFERENCES

Abadias M, Benabarre A, Teixidó N, Usall J, Viñas I (2001). Effect of freeze drying and protectants on viability of the biocontrol yeast Candida sake. Int. J. Food Microbiol. 65(3):173-82.

Abdelwahed W, Degobert G, Stainmesse S, Fessi H (2006). Freeze-drying of nanoparticles: formulation, process and storage considerations. Adv. Drug Deliv. Rev. 58(15):1688-713.

Ayeni FA, Sánchez B, Adeniyi BA, de Los Reyes-Gavilán CG, Margolles A, Ruas-Madiedo P (2011). Evaluation of the functional potential of Weissella and Lactobacillus isolates obtained from Nigerian traditional fermented foods and cow's intestine. Int. J. Food Microbiol. 147(2):97-104.

Brinques GB, Ayub MA (2011). Effect of microencapsulation on survival of Lactobacillus plantarum in simulated gastrointestinal conditions, refrigeration, and yogurt. J. Food Eng. 103(2):123-128.

Burgain J, Gaiani C, Linder M, Scher J (2011). Encapsulation of probiotic living cells: From laboratory scale to industrial applications. J. Food Eng. 104(4):467-483.

Carvalho A, Silva J, Ho P, Teixeira P, Malcata F, Gibbs P (2003). Protective effect of sorbitol and monosodium glutamate during storage of freeze-dried lactic acid bacteria. Le Lait 83(3):203-210.

Carvalho A, Silva J, Ho P, Teixeira P, Malcata F, Gibbs P (2004). Relevant factors for the preparation of freeze-dried lactic acid bacteria. Int. Dairy J. 14(10):835-847.

Chan ES, Wong SL, Lee PP, Lee JS, Ti TB, Zhang Z, Yim ZH (2011). Effects of starch filler on the physical properties of lyophilized calcium–alginate beads and the viability of encapsulated cells. Carbohydr. Polym. 83(1):225–232.

Chang XL, Chen BY, Feng YM (2011). Water-soluble polysaccharides isolated from skin juice, gel juice and flower of Aloe vera Miller. J. Taiwan Inst. Chem. Eng. 42(2):197–203.

Chávez BE, Ledeboer AM (2007). Drying of Probiotics: Optimization of Formulation and Process to Enhance Storage Survival. Drying Technol. 25(7-8): 1193-1201.

De Giulio B, Orlando P, Barba G, Coppola R, Rosa M, Sada A, Nazzaro F (2005). Use of alginate and cryo-protective sugars to improve the viability of lactic acid bacteria after freezing and freeze-drying. World J. Microbiol. Biotechnol. 21(5): 739-746.

De Man J, Rogosa M, Sharpe E (1960). A medium for the cultivation of Lactobacilli. J. Appl. Bacteriol. 23(1):130-135.

Djeraba A, Quere P (2000). In vivo macrophage activation in chickens with Acemannan, a complex carbohydrate extracted from Aloe vera. Int. J. Immunopharmacol. 22(5): 365-72.

Doherty SB, Gee VL, Ross RP, Stanton C, Fitzgerald G, Brodkorb A (2011). Development and characterisation of whey protein micro-beads as potential matrices for probiotic protection. Food Hydrocoll. 25(6):1604-1617.

Doherty SB, Wang L, Ross R, Stanton C, Fitzgerald G, Brodkorb A (2010). Use of viability staining in combination with flow cytometry for rapid viability assessment of Lactobacillus rhamnosus GG in complex protein matrices. J. Microbiol. methods 82(3): 301-10.

Femenia A , García-Pascual P, Simal S, Roselló C (2003). Effects of heat treatment and dehydration on bioactive polysaccharide acemannan and cell wall polymers from Aloe barbadensis Miller. Carbohydr. Polym. 51:397-405.

Fu N, Chen XD (2011). Towards a maximal cell survival in convective thermal drying processes. Food Res. Int. 44(5):1127-1149.

Gbassi GK, Vandamme T, Ennahar S, Marchioni E (2009). Microencapsulation of *Lactobacillus plantarum* spp in an alginate matrix coated with whey proteins. Int. J. Food Microbiol. 129(1):103-5.

Heidebach T, Först P, Kulozik U (2010). Influence of casein-based microencapsulation on freeze-drying and storage of probiotic cells. J. Food Eng. 98(3):309-316.

Hogan SA, McNamee BF, O'Riordan ED, O'Sullivan M (2001). Micro-encapsulating properties of sodium caseinate. J. Agric. Food Chem. 49 (4):1934-1938.

Huang L, Lu Z, Yuan Y, Lü F, Bie X (2006). Optimization of a protective medium for enhancing the viability of freeze-dried Lactobacillus delbrueckii subsp. bulgaricus based on response surface methodology. J. Ind. Microbiol. Biotechnol. 33(1):55-61.

Kailasapathy K (2006). Survival of free and encapsulated probiotic bacteria and their effect on the sensory properties of yoghurt. LWT - Food Sci. Technol. 39(10):1221-1227.

Kanmani P, Satish Kumar R, Yuvaraj N, Paari KA, Pattukumar V, Arul V (2011). Effect of cryopreservation and microencapsulation of lactic acid bacterium Enterococcus faecium MC13 for long-term storage. Biochem. Eng. J. 58-59:140-147.

Khoramnia A, Abdullah N, Liew SL, Sieo CC, Ramasamy K, Ho YW (2011). Enhancement of viability of a probiotic Lactobacillus strain for poultry during freeze-drying and storage using the response surface methodology. Anim. Sci. J. 82(1):127-35.

Lee KW, Park JY, Jeong HR, Heo HJ, Han NS, Kim JH (2012). Probiotic properties of Weissella strains isolated from human faeces. Anaerobe 18(1):96-102.

Manojlović V, Nedovic VA, Kailasapathy K, Zuidam N (2010). Encapsulation of probiotics for use in food products. In N. Zuidam & V. Nedović (Eds.), Encapsulation technologies for active food ingredients and food processing. New York: Springer.

Martínez-Romero D, Alburquerque N, Valverde JM, Guillén F, Castillo S, Valero D, Serrano M (2006). Postharvest sweet cherry quality and safety maintenance by Aloe vera treatment: A new edible coating. Postharvest Biol. Technol. 39(1):93-100.

Meng XC, Stanton C, Fitzgerald GF, Daly C, Ross RP (2008). Anhydrobiotics: The challenges of drying probiotic cultures. Food Chem. 106(4):1406-1416.

Miranda M, Vega-Gálvez A, García P, Di Scala K, Shi J, Xue S, Uribe E (2010). Effect of temperature on structural properties of Aloe vera (Aloe barbadensis Miller) gel and Weibull distribution for modelling drying process. Food Bioproducts Process. 88(2-3):138-144.

Nam H, Ha M, Bae O, Lee Y (2002). Effect of Weissella confusa Strain PL9001 on the Adherence and Growth of Helicobacter pylori. Appl. Environ. Microbiol. 68(9):4642-4645.

Nanasombat S, Sriwong N (2007). Improving viability of freeze-dried lactic acid bacteria using lyoprotectants in combination with osmotic and cold adaptation. KMITL Sci. Technol. J. 7(S1): 61-69.

Nedovic V, Kalusevic A, Manojlovic V, Levic S, Bugarski B (2011). An overview of encapsulation technologies for food applications. Procedia Food Sci. 1: 1806-1815.

Ni Y, Turner D, Yates KM, Tizard I (2004). Isolation and characterization of structural components of Aloe vera L. leaf pulp. Int. Immunopharmacol. 4(14):1745-55.

Nindo CI, Powers JR, Tang J (2010). Thermal properties of aloe vera powder and rheology of reconstituted gels. Trans. ASABE 53(4):1193-1200.

Otero MC, Espeche MC, Nader-Macías ME (2007). Optimization of the freeze-drying media and survival throughout storage of freeze-dried Lactobacillus gasseri and Lactobacillus delbrueckii subsp. delbrueckii for veterinarian probiotic applications. Process Biochem. 42(10): 1406-1411.

Picot A, Lacroix C (2004). Encapsulation of bifidobacteria in whey protein-based microcapsules and survival in simulated gastrointestinal conditions and in yoghurt. Int. Dairy J. 14(6):505-515.

Reddy KB, Awasthi SP, Madhu AN, Prapulla SG (2009). Role of Cryoprotectants on the Viability and Functional Properties of Probiotic Lactic Acid Bacteria during Freeze Drying. Food Biotechnol. 23(3): 243-265.

Reynolds T, Dweck AC (1999). Aloe vera leaf gel: a review update. J. Ethnopharmacol. 68(1-3):3-37.

Semyonov D, Ramon O, Kaplun Z, Levin-Brener L, Gurevich N,

Shimoni E (2010). Microencapsulation of Lactobacillus paracasei by spray freeze drying. Food Res. Int. 43(1): 193-202.
Serna L, Valencia JL, Campos R (2010). Cinética de fermentación y acción antimicrobiana de Weissella confusa contra Staphylococcus aureus y Streptococcus agalactiae. Rev. Fac. Ing. Univ. Antioquia 55: 55-65.
Serna-Cock L, Rubiano-Duque LF, Loaiza-Castillo NB, Enríquez-Valencia CE (2012). Weissella confusa como un agente bioprotector en la inocuidad alimentaria contra patógenos Gram negativos. Alimentos Hoy 21(27):102-114.
Serna-Cock L, Vallejo-Castillo V (2013). Probiotic encapsulation. Afr. J. Microbiol. 7(40):4743-4753.
Shui G, Leong LP (2006). Residue from star fruit as valuable source for functional food ingredients and antioxidant nutraceuticals. Food Chem. 97(2):277-284.
Song CS, Nam JH, Kim CJ, Ro ST (2005). Temperature distribution in a vial during freeze-drying of skim milk. Journal of Food Engineering, 67(4): 467–475.
WHO/FAO (2006). Probiotics in food. Health and Nutritional Properties and Guidelines for Evaluation: FAO Food and Nutrition Paper, 85. Rome.
Yang C, Zhu X, Fan D, Mi Y, Luo Y, Hui J, Su R (2012). Optimizing the Chemical Compositions of Protective Agents for Freeze-drying Bifidobacterium longum BIOMA 5920. Chin. J. Chem. Eng. 20(5):930-936.
Zayed G, Roos YH (2004). Influence of trehalose and moisture content on survival of Lactobacillus salivarius subjected to freeze-drying and storage. Process Biochem. 39(9):1081-1086.

academicJournals

Vol. 13(26), pp. 2668-2674, 25 June, 2014
DOI: 10.5897/AJB2014.13838
Article Number: 9A5EBFD45612
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Pigment production from a mangrove *Penicillium*

Lathadevi Karuna Chintapenta[1,2]*, Chandi Charan Rath[3], Bapuji Maringinti [2,4] and Gulnihal Ozbay[1]

[1]Delaware State University, Department of Agriculture and Natural Resources, Dover, DE 19901, USA.
[2]DLR College of PG Courses, Gollala Mamidada, Andhra Pradesh 533344, India.
[3]North Orissa University, Baripada, Orissa 757003, India.
[4]Acharya BMR College of Pharmacy, Soldevanahalli, Bangalore 560107, India.

Received 9 April, 2014; Accepted 2 June, 2014

A mangrove *Penicillium* producing red pigment was cultured in an optimized medium that was designed by the authors previously and used in this study. The purpose of this study was to identify the pigment and also to study the effect of bio elements on pigment production. Pigment from the medium was efficiently extracted using chloroform, ethyl acetate and n-butanol. Most of the red pigment was extracted into ethyl acetate and further purified by preparative thin layer chromatography. From ^{1}H and ^{13}C NMR data supported by electronic imaging mass spectrometry, structure of the compound was elucidated as 2-(4-acetyl phenyl) acetic acid. The yield of pigment produced was studied with respect to various salts and bio elements. Salts at high concentrations (sodium chloride, ammonium sulfate, and sodium nitrite) had a drastic effect on pigment yield because most of the pigment remained adhered to the mycelium instead of diffusing into the medium. Also, when bio elements were supplemented to the medium; calcium, iron, and zinc enhanced pigment yield whereas; potassium, magnesium, copper and manganese did not have significant impact on pigment production. Lead had a drastic negative effect on the pigment yield. Therefore, this study proves that salts and bio elements play a major role in the production of various metabolites from mangrove fungi.

Key words: *Penicillium*, 2-(4-acetyl phenyl) acetic acid, bio elements, salts, soluble pigment.

INTRODUCTION

Fungi have been the source of many important anti-bacterial agents including penicillin's and cephalosporins, both of which have been used heavily for the past 50 to 60 years. Overall, many metabolites are being produced by *Aspergillus* and *Penicillium* species which are salt tolerant, fast growing species and are easily obtained from many substrates (Bugni and Ireland, 2004; Isaka et al., 2000; Park et al., 1999; Udagawa et al., 2000). Marine fungi are well known sources for novel biologically active secondary metabolites (Bugni and Ireland, 2004) and have been found to produce different metabolites compared to terrestrial organisms (Spery et al., 1998). Pigments from natural sources are one group of industrially significant metabolites, because of the negative impacts artificial synthetic colorants have on human health. Natural pigments have widely been used

*Corresponding author. E-mail: kchintapenta@desu.edu. Tel: 1-302-857-6405. Fax: 1-302-857-6402

in foodstuff, cosmetics, and pharmaceutical manufacturing processes (Francis, 1987; Kim et al., 1995). Microbial pigments can become highly significant when the production yield is high and the pigments are highly stable (Cho et al., 2002).

The ocean environment is a rich source for novel metabolite producing microbes, but only during last two decades the studies on marine metabolites have increased. Some previous studies showed that the marine *Penicillium* produced pigments (PP-V and PP-R) and these are similar in structure to the pigments produced from *Monascus* (monascorubrine and monascuscorubramine) (Ogihara and Oishi, 2002); pigments from *Monascus* are being used in the food industry since long time. Mohan and Vijay-Raj (2009) also described pigment production and radical scavenging activity from a *Penicillium* sp NIOM-2 isolated from marine sediment in India. Three other halophilic fungal strains *Hortaea werneckii*, *Phaeotheca triangularis*, and *Trimmatostroma salinum* isolated from the salterns in the eastern coast of the Adriatic Sea, produced melanin pigments at saturated concentrations of sodium chloride (Tina et al., 2006). Details of several other studies on marine fungi producing significant metabolites are seen but literature on industrial application of potential marine fungal pigments is very minimal and this drives our interest to study the pigment potential of a mangrove *Penicillium*.

The purpose of this research was to isolate fungal pigments from mangrove sediments because mangroves exist under conditions of high salinity, extreme tides, high temperature, and various other stress conditions (Kathiresan and Bingham, 2001). Therefore microbes growing under these conditions will have the potential to produce different significant metabolites to cope with these stresses.

Research on mangrove pigments is very less; it has been recorded that microorganisms from mangroves open up new areas for potential biotechnological exploitation (Gopal and Chauhan, 2006). During this study period, we isolated nearly 100 mangrove fungi from Godavari mangroves of India. Most of the fungi were pigment producers and a red pigment-producing *Penicillium* was selected to study the pigment, its optimization conditions and the effect of metals and salts on pigment yield. In this paper, pigment extraction method, pigment structure and the impact of different bio elements and salts on pigment yield were explained. The main objective of this study was to identify which bio elements have a positive effect on pigment production and also to study if salts effect pigment production even when the fungus was from a marine origin.

MATERIALS AND METHODS

Microorganism and inoculum preparation

The mangrove fungus DLR-7 isolated from Godavari Delta, Andhra Pradesh, India was identified as *Penicillium* sp. according to Alexopolus and Mims (1979). Stock cultures of (Karuna et al., 2009) this *Penicillium* were maintained on potato dextrose agar slants prepared with 50% aged seawater; the cultures were revived every month and stored at 4°C until used in the experiment. Inoculum for these studies was prepared by growing the fungus initially at 25°C on potato dextrose agar (PDA) plates for seven days. Plates having uniform growth and sporulation were selected and a 0.7 cm^2 plug from the outer zone of the colony was punched with a sterile cutter. The plugs were transferred to 100 ml of culture medium in 250 ml flasks and incubated under static conditions at 25°C until maximum pigment was produced (Gunasekaran and Poorniamaal, 2008).

Culture conditions

An optimized basal culture medium was designed for red pigment production using potato extract prepared in the laboratory. Two hundred grams of potatoes were cleaned, sliced and cooked for 30 min with 500 ml of distilled water; the cooked potato slices were mashed and the liquid was filtered through a muslin cloth (Aneja, 2003). Then, 500 ml of seawater was added to the medium and autoclaved. Optimization of culture medium was carried out using various carbon and nitrogen sources and altering different physical parameters (Lathadevi et al., 2014). Results from the optimization experiments concluded that xylose (2% w/v) and glycine (1% w/v) when supplemented to potato extract and pH adjusted to 3.0, produced a high yield of pigment. Therefore, this medium was further used to extract and identify the pigment. The medium was also supplemented with different salts and bio elements to study if they have any effect on the concentration of the pigment produced.

Extraction of pigment

Extraction of pigment from the liquid culture media was carried out by different solvents viz. non polar to polar (Padmavathi and Prabhudessai, 2013). Chloroform, ethyl acetate, and n-butanol were used for the extraction process; all the solvents used were obtained from Qualigens Fine Chemicals Pvt. Ltd., (Mumbai, India). Five liters of culture medium were prepared and about 500 mL were dispersed in ten 1 L conical flasks. The media were autoclaved and about 0.7 cm^2 plugs from the outer zone of the *Penicillium* culture plates were transferred to the media. The inoculated flasks were incubated under static conditions at 25°C (Gunasekaran and Poorniamaal, 2008) for 12 days and the cultures were then harvested. The culture medium was then passed through filter paper (No. 1; Whatman, India Liasion Office, Mumbai, India). A three stage multi-contact/counter current extraction method was used to extract the pigment. Three 500 ml separating flasks were used, 200 ml of the filtered broth was added to the flasks, and 100 ml of distilled solvent was added to the first flask, shaken well and allowed to stand until the aqueous and organic layers separated. Organic layer was transferred to the second flask treated as in the previous step and repeated with the third flask as shown in Figure 1.

Finally, solvent with the pigment was transferred to a clean conical flask and extraction was repeated until no more pigment diffused into the solvent. The entire culture broth was extracted in the same way with chloroform, ethyl acetate, and n-butanol. Finally, the solvents were stripped off with the help of a rotary vacuum evaporator and the amount of pigment was weighed and purified by chromatography. Absorbance of the extracts was measured using a UV-visible spectrophotometer (Model 117, Systronics, India).

Chromatographic analysis

Thin layer chromatography (TLC, Sigma-Aldrich, Hyderabad, India),

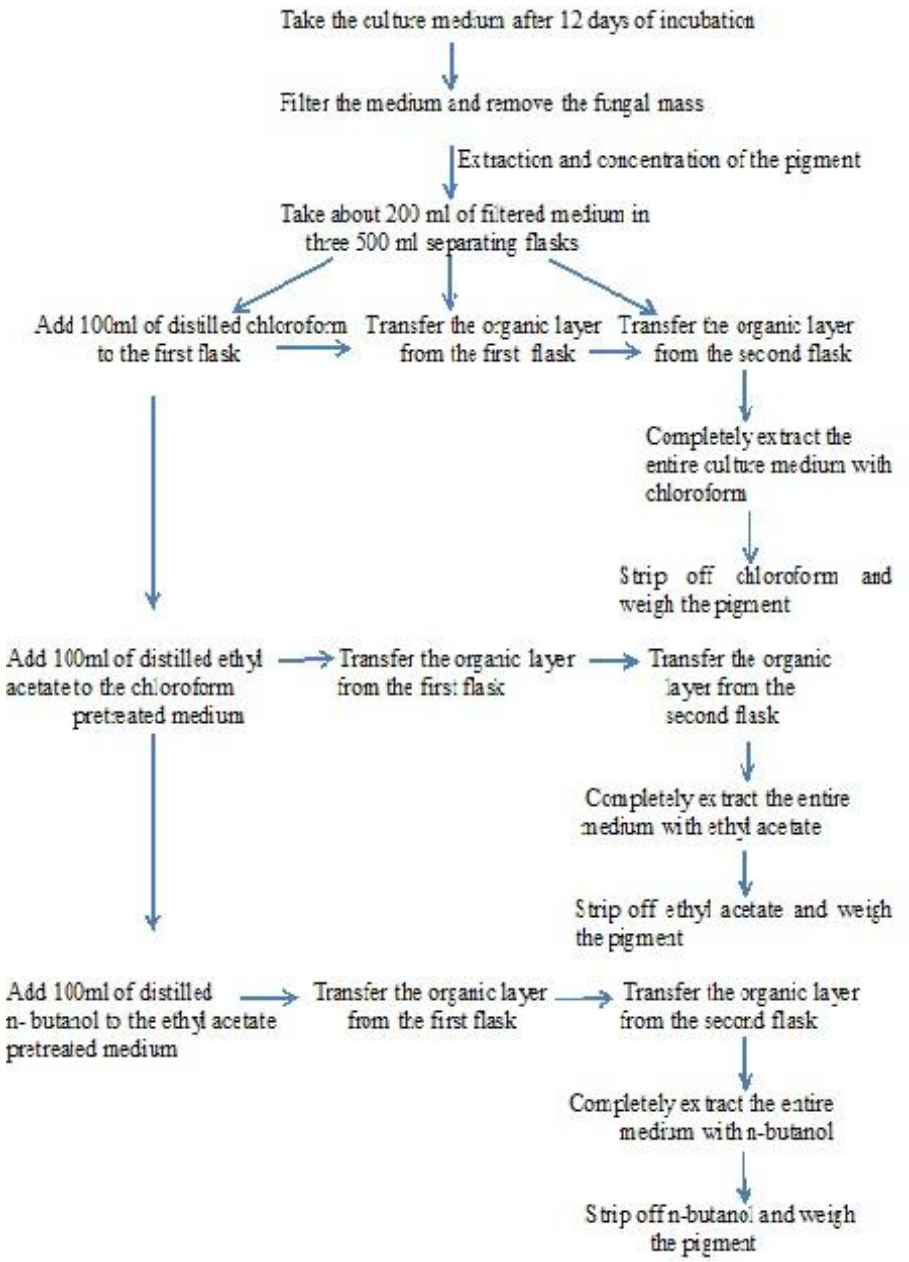


Figure 1. Extraction of pigment from the culture medium: This figure explains the process how pigment is being extracted from the culture medium.

was performed to study the number of pigment components present in each crude pigment fraction obtained from solvent extraction. The ethyl acetate fraction had more pigment but fewer pigment components, which were further purified by preparative TLC using chloroform: methanol (1:1). The pigment was later identified by nuclear magnetic resonance spectroscopy (NMR, Jeol 400 MHZ, USA).

Effect of salts on pigment production

The culture medium was prepared as described in the earlier section and about 50 ml of the medium was dispersed into 100 ml conical flasks. These flasks were then treated with different concentrations of sodium chloride (0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0 and 10% w/v), sodium nitrite, and ammonium sulfate separately and adjusted to pH 3.0. The media were then autoclaved and inoculated with the *Penicillium* cultures as described earlier. This study was performed to determine if salts in the culture medium affect the concentration of pigment produced. All the experiments with salts were performed in triplicates and compared to a control (media not treated with salts).

Effect of bio elements on pigment production

This experiment was designed to study the effect of bio elements on pigment production. The culture medium was supplemented with

Table 1. Characteristics of the pigment fractions after solvent extraction.

Fraction	Color	λmax (nm)	Weight (mg/L)
CF	Light Yellow	450 470 510	70
EaF	Deep Red	450 470 510	600
BF	Red	510	250

CF, Chloroform fraction; EaF, Ethyl acetate fraction; BF, Butanol fraction.

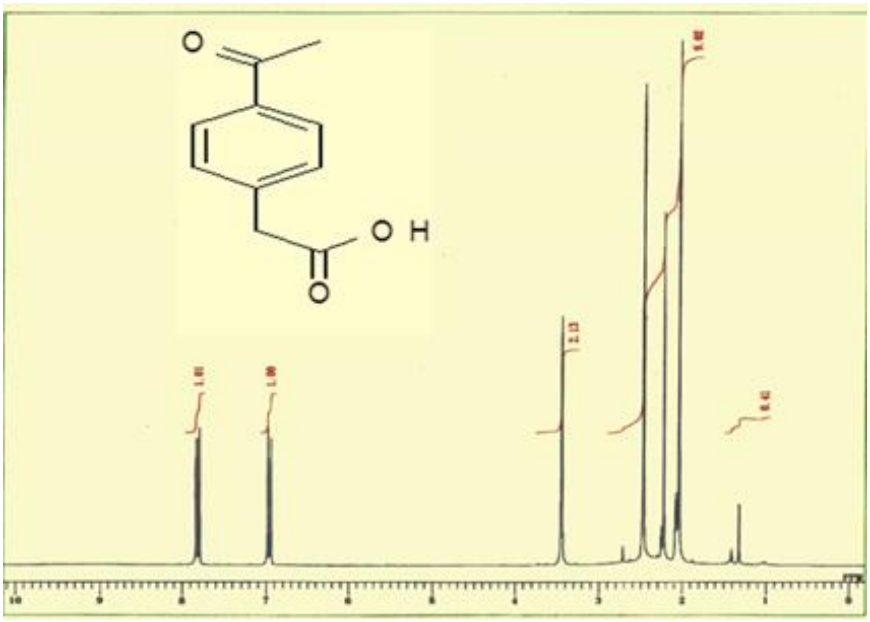


Figure 2. ^{1}HNMR spectrum of the compound [2-(4-acetyl phenyl) acetic acid] in $CDCl_3$.

different concentrations of calcium sulfate ($CaSO_4 \cdot 2H_2O$), copper sulfate ($CuSO_4 \cdot 5H_2O$), ferrous sulfate ($FeSO_4 \cdot 7H_2O$), potassium chloride (KCl), lead nitrate ($PbNO_3$), magnesium sulfate ($MgSO_4 \cdot 7H_2O$), manganese sulfate ($MnSO_4 \cdot H_2O$), and zinc sulfate ($ZnSO_4$). These trace metal concentrations were used at concentrations typically found in sea water. Stock solutions ranging from 10^5 to 10^6 ppb were prepared for calcium, magnesium and potassium. From the stock, different working concentrations (10^5 ppb to 15×10^5 ppb) were prepared and added to the culture media. For metals such as iron, copper, manganese, lead and zinc, a pre-stock solution was prepared and the required volumes were taken to obtain the respective concentrations of 0.05 ppb to 1 ppb. 50 ml of media was prepared with pH 3.0, sterilized and inoculated with the test organism as described above and incubated at 25°C for 12 days. All experiment treatments were performed in triplicates and compared to control media (media without trace elements).

Statistical analysis

Data analysis for treatments with salts and trace elements was performed to study their effect on pigment production. Multiple comparative analyses were performed using R version 2.15.3 for Statistical Computing (http://www.r-project.org/). Linear regression function “lm ()” in R was used to determine significant differences between the treatments using {lm (formula = y ~ x)}. Treatments statistically significant ($p < 0.05$) are considered to be positively affecting pigment production. Regression coefficients and p values of significant treatment comparisons were depicted in the table.

RESULTS AND DISCUSSION

The absorption maxima (λmax) and weight of the pigment fractions derived by solvent extraction are presented in Table 1. The chloroform fraction was colorless whereas, the ethyl acetate and n-butanol fractions were deep red. Chloroform and ethyl acetate extracts had fewer components and were fairly pure, but the butanol fraction had a large number of compounds. As the primary interest of this research was to investigate the red pigment, the ethyl acetate fraction was selected and further purified to elucidate the structure of the red pigment. Preparative TLC of the ethyl acetate fraction yielded a crystalline compound with a melting point of 156°C. It had a strong blue fluorescence on the TLC plate under UV; however, it appeared as a green arrow headed spot after spraying with 5% sulfuric acid in methanol. From ^{1}H, ^{13}C nuclear magnetic resonance (NMR) data supported by electronic impact mass spectroscopy (EIMS) , the structure of the compound was elucidated as [2-(4-acetyl phenyl) acetic acid] as presented in Figure 2. However, during the chromatographic separation, the compound turned out to be colorless.

The ^{1}H NMR spectrum of the compound gave signals

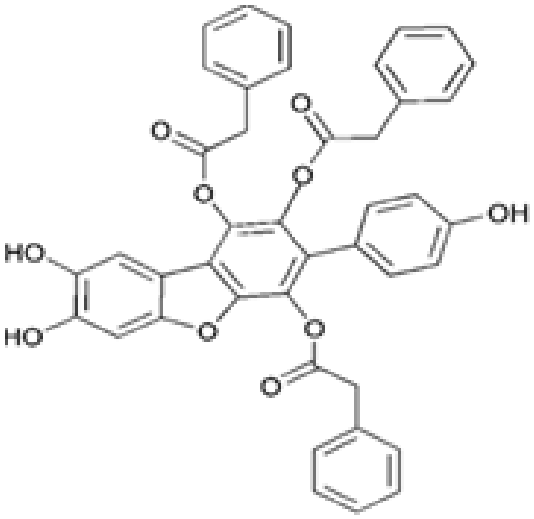


Figure 3. Structure of Ganbajunin-B: This is the assumed structure of the parent molecule from which the pigment component is derived.

at δ 7.82 (2H, d, *J*=7.5Hz), 6.92 (2H, d, *J* = 7.5Hz), 3.45 (2H, s), and 2.2 (3H, s) as shown in Figure 2. High resolution proton magnetic resonance (PMR) showed a pair of doubles in the aromatic region. From the foregoing spectral data, structure of the compound was established as 2-(4-acetylphenyl) acetic acid. ^{13}C NMR supported the structure assigned: δ 22.4, 41.6, 128.7(2C), 129.7 (2C), 135.5, 135.9, 178.4 and 196.2. Further, the structure was confirmed by EIMS, which displayed molecular ion at *m/z* 178. From the literature the parent molecule of this compound was emphasized to be Ganbajunin B that has a red color (Figure 3).

Effect of salts on pigment production

When the fungal cultures were exposed to different salts (as we listed them in the method section) under optimized culture conditions, the soluble red pigment that was supposed to diffuse into the medium, was essentially absent, especially when sodium nitrite and ammonium sulfate were present in the medium. These electrolytes might have altered the pH and prevented diffusion of the pigment, since in the control without salts, the pigment was soluble. In media containing sodium chloride at various concentrations, there was a soluble red pigment but the concentration of pigment was less than that of the control. When the concentration of sodium chloride increased to more than 2% w/v, the concentration of pigment produced was less. Pigment was produced at a significant concentration in medium containing sodium chloride at 1.5% w/v as shown in Table 2. Secondary metabolite composition and production varies with salt concentration. Halo tolerant marine fungal species have evolved unique metabolic mechanisms in response to salt concentrations. In the marine environment, these fungi must have osmoregulatory mechanisms since the biosynthesis of solutes for osmoregulation is energetically costly. Fungi may exhibit decreased secondary metabolite production/slower rates of metabolite production in the presence of high salt concentrations. These findings suggest that marine-derived fungal metabolite production is modulated by the salt concentration of sea water (Bugni et al., 2003). When the sodium chloride concentration increased from 8 to 10% w/v, pigment solubility decreased where most of the pigment was present in the mycelia. High salt conditions also affected the fungal spores because brown spores were observed instead of red.

Effect of trace elements on pigment production

Several studies reported that trace elements are important factors affecting pigment production in several microorganisms (Kim et al., 1998; An et al., 2001). Calcium at 10^5 ppb increased the pigment yield when compared to the control. But increased calcium concentrations, such as $6x10^5$ ppb, inhibited sporulation and decreased pigment production. Potassium at $2×10^5$ ppb produced less pigment than the control. This may be because potassium plays an important role in ionic balance (osmoregulation), enzyme activity and cell physiology (Kavanagh, 2011). Therefore potassium may be used for fungal growth but does not enhance pigment production. The treatments with magnesium, copper, and manganese also had less pigment than the control groups. Zinc and iron stimulated pigment production when compared to the control. Lead at concentrations of 50 ppb inhibited pigment production and at lower concentrations did not have a positive effect on the pigment yield when compared to the control. Studies by Cuero and Ouellet (2005) demonstrated that metal ions such as zinc, copper, and iron have a stimulatory effect on fungal growth and gene expression.

Iron is thought to be necessary for both primary and secondary metabolism. In particular, iron is a constituent of the active centres of the cytochrome P-450 of various mono oxygenases involved in the biosynthesis of clavine alkaloids (Boonyapranai et al., 2008). Metal ions such as zinc, iron,and copper have a regulatory effect at the cellular and molecular levels, on fungal growth and metabolite synthesis. However, the effect depends on the type and concentration of the metal ions, as well as on the presence of ligands in the substrate and/or host (Weinberg, 1977; Hughes and Poole, 1989; Cuero, 2001). Studies by Cuero and Ouellet (2004), demonstrated a stimulatory effect of the metal ions zinc, copper, and iron on gene expression, growth and metabolite production by phyto pathogenic fungi.

In this study trace elements such as calcium, zinc, iron,

Table 2. Comparative regression analysis of the treatments and control on pigment production.

Substrate	Comparison	Estimate of difference	Standard Error	t value	P-Value
Sodium chloride (%)	1.5*2.0	3.03	0.10	-29.99	$1.72x10^{-15}$
	1.5*Control	-10.33	0.10	102.24	$< 2x10^{-16}$
Calcium (ppb)	10^5*$5x10^4$	12.22	0.11	-103.27	$< 2x10^{-16}$
	10^5*Control	4.87	0.11	-41.15	$2.74x10^{-14}$
Potassium (ppb)	$2x10^5$*10^5	4.03	0.11	-33.96	$2.7x10^{-13}$
	2 $x10^5$*Control	-8.51	0.11	71.72	$< 2x10^{-16}$
Magnesium (ppb)	$15x10^5$*10^6	4.12	0.10	-37.55	$1.87x10^{-15}$
	$15x10^5$*Control	-16.82	0.10	153.30	$< 2x10^{-16}$
Iron (ppb)	0.5*1	13.7	0.12	-108.45	$< 2x10^{-16}$
	0.5*Control	0.61	0.12	-4.82	0.0004
Copper (ppb)	0.5*1	0.36	0.11	-3.02	0.0106
	0.5*Control	-19.24	0.11	161.39	$< 22x10^{-16}$
Manganese (ppb)	0.1*2	7.7	0.11	-64.95	$< 2x10^{-16}$
	1*Control	-13.24	0.11	111.68	$< 2x10^{-16}$
Lead (ppb)	0.01*0.05	-10.59	0.14	-73.46	$1.31x10^{-12}$
	0.01*Control	-8.29	0.14	57.50	$9.29 x10^{-12}$
Zinc (ppb)	100*50	0.09	0.13	-0.65	0.524
	100*Control	4.27	0.13	-31.14	$7.56 x10^{-13}$

*: indicates comparison; +: P > 0.05 is not significant for the treatment comparisons. Estimate of difference is the difference of sample means and control means.

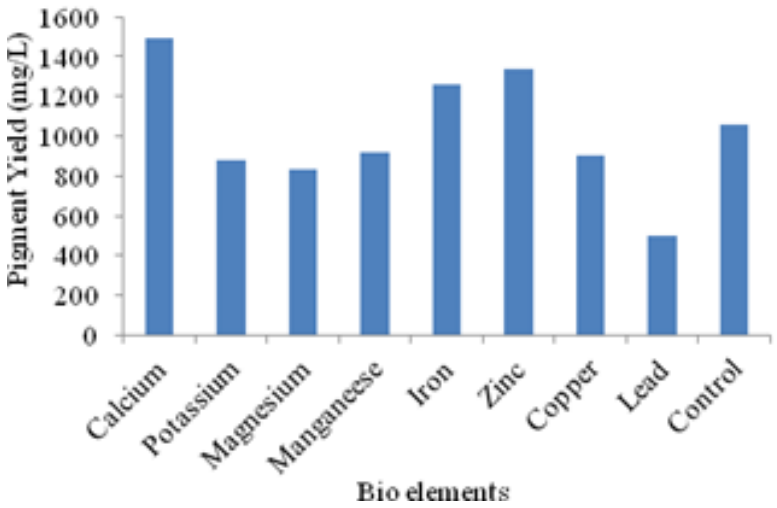


Figure 4. Effect of bio elements on pigment yield: This graph explains how different bio elements affect pigment yield.

and manganese positively influenced pigment yield when compared to the control (without trace elements). Similar findings were observed by Boonyapranai et al. (2008) (Figure 4) on a soil fungus, *Fusarium verticilloides*. Of all

the trace elements studied, calcium produced maximum pigment (1,490 mg/L) while the control yielded 1,065 mg/L of pigment. Bio elements such as zinc and calcium effect fungal growth and gene expression (Cho et al., 2002).

Limited literature is available on pigments from mangrove fungi. This is the first study providing the investigations on the effect of trace elements on mangrove fungal pigments. Observations recorded will significantly contribute for further work in this area. Pigments from terrestrial fungi like *Monascus* are being industrially used in the food industry, but there are no studies on the industrial use of pigments from mangrove fungi. The pigment component 2-(4-acetylphenyl) acetic acid isolated during this study is similar to Ganbajunin B, which is a brownish red pigment isolated from a mushroom *Thelephora vialis.* This compound has strong antioxidant properties and this mushroom is a favorite food in China due to its strong flavor and taste.

The pigment component isolated from mangrove fungus in this study has significant characters such as the compound isolated from the mushroom (*T. vialis)* and further detailed studies such as toxicity tests are required. These studies will be helpful to explore the industrial uses of mangrove fungal pigments.

Conflict of Interests

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

ACKNOWLEDGMENTS

We would like to thank Mr. Vivekananda Reddy, DLR College, Gollala Mamidada, India, for the financial support and use of research facilities. Special thanks to Dr. Liang Liu, University of Georgia for advice on statistical analysis and interpretation of the research results. Thanks to Dr. Gary Richards, U.S. Department of Agriculture, ARS, Dover, Delaware for his critical review of the manuscript. We also thank the USDA-NIFA CBG 1890 Water Resource Center Program for their assistance with the publications costs of this article.

REFERENCES

Alexopolus CJ, Mims CW (1979). Introductory mycology, 3rd ed., Wiley Eastern Limited, New Delhi. pp. 189- 470.

An GH, Jang BG, Suh OS, Kim CJ, Song KB (2001). Iron (III) decreases astaxanthin production in *Phaffia rhodozyma* (*Xanthopyllomyces dendrorhous*). Food Sci. Biotechnol. 10 (2):204-207.

Aneja KR (2003). Experiments in microbiology, plant pathology and biotechnology, 4th ed., New Age International Publishers, New Delhi. pp. 632-635.

Boonyapranai K, Tungpradit R, Lhieochaiphant S, Phutrakul S (2008). Optimization of submerged culture for the production of naphthoquinone pigment by *Fusarium verticillioides.* Chiang Mai J. Sci. 35(3):457-466.

Bugni TS, Bernan VS, Greenstein M, Janso JE, Maiese WM, Mayne CL, Ireland CM (2003). Brocaenols A-C: novel polyketides from a marine derived *Penicillium brocae.* J. Org. Chem. 68 (5):2014-2017.

Bugni TS, Ireland CM (2004). Marine-derived fungi: a chemically and biologically diverse group of microorganisms. Nat. Prod. Rep. 21:143-163.

Cho JPP, Wang HJH, Kim SW, Cho JW, Yun JW (2002). Production of red pigment by submerged culture of *Paecilomyces sinclairii*. Lett. Appl. Microbiol. 35(3):195-202.

Cuero R (2001). Regulation of mycotoxins formation and fungal growth by metal ions and fertilizer: effect on fungal gene expression, In: De Koe WJ, Samson RA, Van-Egmond HP, Gilbert J and Sabioino M (Eds.). Mycotoxins and phycotoxins in perspective at the turn of the millennium. Proceedings of the 10th International IUPAC symposium on mycotoxins and phycotoxins, Guaruja, Brazil. pp. 21-25.

Cuero R, Ouellet T (2005). Metal ions modulate gene expression and accumulation of the mycotoxins aflatoxin and zearalenone. J. Appl. Microbiol. 98 (3):598-605.

Francis FJ (1987). Lesser known food colorants. Food Technol. 41:62-68.

Gopal B, Chauhan M (2006). Biodiversity and its conservation in the Sundarban Mangrove Ecosystem. Aquat. Sci. 68:338-354.

Gunasekaran S, Poorniammal R (2008). Optimization of fermentation conditions for red pigment production from *Penicillium* sp. under submerged cultivation. Afr. J. Biotechnol. 7 (12):1894-1898.

Hughes M, Poole RK (1989). Metal mimicry and metal limitation in studies of metal-microbe interactions. In: Poole RK, Gadd GM (Eds.). Metal-microbe interactions, Oxford; IRL Press, New Delhi, India. pp. 1-17.

Isaka M, Jaturapat A, Kladwang W, Punya J, Lertwerawat Y, Tanticharoen M, Thebtaranonth Y (2000). Antiplasmodial compounds from the wood-decayed fungus *Xylaria* sp. BCC 1067. Plan. Med. 66: 473-475.

Karuna ChLD, Bapuji M, Rath CC, Murthy YLN (2009). Isolation of mangrove fungi from Godavari and Krishna delta of Andhra Pradesh, India. J. Ecobiol. 24(1):91-96.

Kathiresan K, Bingham BL (2001). Biology of mangroves and mangrove ecosystems. Adv. Mar. Biol. 40:81-251.

Kavanagh K (2011). Fungi-Biology and Applications, 2nd ed., Wiley-Blackwell publications, United Kingdom. pp. 257-279.

Kim CH, Kim SW, Hong SI (1998). Production of red pigment by *Serratia* sp. KH-95 and its cultural properties. Korean J. Biotechnol. Bioeng. 13:431-437.

Kim JK, Park SM, Lee SJ (1995). Novel anti mutagenic pigment produced by *Bacillus licheniformis* SSA3. J. Microbiol. Biotechnol. 5: 48-50.

Lathadevi KCh, Chandi CR, Bapuji M, Gulnihal O (2014). Culture conditions for growth and pigment production of a mangrove *Penicillium* species. J. Multidisc. Res. 1(1):29-35.

Mohan AD, Vijay-Raj AS (2009). Pigment and amylase production in *Penicillium* sp. NIOM-02 and its radical scavenging activity. Int. J. Food Sci. Technol. 44(12):2424-2430.

Ogihara J, Oishi K (2002). Effect of ammonium nitrate on the production of PP-V and monascorubrin homologues by *Penicillium* sp. AZ. J. Biosci. Bioeng. 93:54-59.

Padmavathi T, Prabhudessai T (2013). A solid liquid state culture method to stimulate *Monascus* pigments by intervention of different substrates. Int. Res. J. Biol. Sci. 2(10):22-29.

Park JS, Lee KR, Kim JC, Lim SH, Seo JA, Lee YW (1999). A hemorrhagic factor (apicidin) produced by toxic *Fusarium* isolates from soybean seeds. Appl. Environ. Microbiol. 65 (1):126-130.

Spery S, Gary JS, Phillip C (1998). Vertinoid polyketides from the salt water culture of the fungus *Trichoderma longibrachiatum* separated from a Halicona marine sponge. J. Org. Chem. 63(26):1011-1014.

Tina K, Michael HW, Tea LR, Nina GC (2006). Evidence for 1,8-dihydroxy naphthalene melanin in three halophilic black yeasts grown under saline and non- saline conditions. FEMS Microbiol. Lett. 232(2):203-209.

Udagawa T, Yuan J, Panigraphy D, Chang YH, Shah J, D'Amato, J (2000). Cytochalasin E: an epoxide containing *Aspergillus* derived fungal metabolite, inhibits angiogenesis and tumor growth. J. Pharmacol. Exp. Ther. 294:421-427.

Weinberg ED (1977). Mineral element control of microbial secondary metabolism. In: Weinberg E (Ed.). Microorganisms and minerals, Vol.3, marcel Dekker, New York. pp. 289-316.

academicJournals

Vol. 13(26), pp. 2675-2678, 25 June, 2014
DOI: 10.5897/AJB2014.13754
Article Number: 844272A45613
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Total aflatoxin, fumonisin and deoxynivalenol contamination of busaa in Bomet county, Kenya

Mary C. Kirui[1]*, Amos E. Alakonya[1], Keith K. Talam[1], Gonoi Tohru[2] and Christine C. Bii[3]

[1]Jomo Kenyatta University of Agriculture and Technology, Institute of Biotechnology Research, Kenya.
[2]Chiba University Medical Mycology Research Centre-Japan, Japan.
[3]Kenya Medical Research Institute, Mycology laboratories, Centre for Microbiology Research, Kenya.

Received 24 February, 2014; Accepted 2 June, 2014

Mycotoxin contamination is a common problem in developing countries, particularly in cereals, and this poses a serious health risk to its consumers. Busaa is a Kenyan traditional brew whose cereal ingredients are prone to mycotoxin contamination. This study aimed at detecting the presence and subsequently quantifying aflatoxin, fumonisin and deoxynivalenol (DON), in busaa in Bomet county, Kenya. Busaa samples were collected from homesteads involved in brewing in the north eastern part of Bomet East constituency. Mycotoxins were detected in the samples using the Envirologix QuickTox kits and quantified using the QuickScan machine according to the manufacturer's instructions. Among the 61 samples tested, 93, 9.8 and 23% were contaminated with aflatoxin, fumonisin and DON, respectively, (mean: 5.2±0.2 µg/kg, range: 2.8 to 11 µg/kg; mean 1460±188 µg/kg, range 280 to 4000 µg/kg, mean 259±5.2 µg/kg, range 200 to 360 µg/kg, respectively). Although traditional brews are not directly included in the European law on mycotoxins, it is important to consider their mycotoxin levels. In this study, busaa is a mainly a maize product and also the European Union (EU) guidelines on mycotoxins in maize were used as reference. It was found out that 65.6% of busaa had aflatoxin levels above the limit set in the EU guideline (4 µg/kg). Although, the average levels of fumonisin and DON were within the set limits (fumonisins: 4000 µg/kg; DON: 1750 µg/kg), studies have shown that chronic exposure to multiple mycotoxins has detrimental health effects. Therefore, there is need for mycotoxicological quality control of traditionally produced brews for public mycotoxicological safety.

Key words: Mycotoxin, traditional brew.

INTRODUCTION

Busaa (maize beer) is a Kenyan traditional brew that has a socio-cultural significance and is mostly consumed during events such as male circumcisions, weddings and funerals. It is also commonly consumed by members of low income earning groups as a leisure activity especially when there is a bumper harvest (Daško et al., 2005). The main ingredients of busaa are raw maize flour and semi-ground finger millet malt. Natural fermentation of the raw maize flour followed by subsequent addition of the semi-ground finger millet malt and farther fermentation results

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

in busaa as the final product. The production and consumption of cereals such as maize, sorghum and millet in Africa is highly constrained by rot fungi including *Aspergillus*, *Fusarium* and *Penicillium* (Milićević et al., 2010; Shephard, 2008; Kedera et al., 1998). These fungi lower the quality of the produce through discoloration and reduction of nutritional value besides production of mycotoxins such as fumonisins, aflatoxins, ochratoxins, deoxynivalenol and zearalenone (Jacobsen, 2008). In animals, aflatoxins have been shown to be carcinogenic, mutagenic, terratogenic and immunosuppressive cause (Ajeyuyo et al., 2011) while fumonisins are generally considered to be the cause of equine leukoencephalomalacia in horses, mules, and donkeys and are strongly associated, based on epidemiology with esophageal cancer and neural tube defects in humans (Jacobsen, 2008; Wakhisi et al., 2005; Paul et al., 2001). On the other hand, the presence of DON in feeds is manifested by rejection of feeds, vomiting, diarrhea and eventual weight loss in livestock (Kuiper-Goodman, 2002). It is expected that cereals contaminated with fungi and mycotoxins are discarded but this is not always the case. Studies have shown that these cereals are sometimes consumed by humans, fed to animals or diverted to alcohol production, resulting in transfer of mycotoxins along the food chain and hence the occurrence of mycotoxicoses (Jacobsen, 2008; Moturi, 2008; Bennett and Klich, 2003; Sudakin, 2003, Binder et al., 2007).

Typically, there are five major stages in the manufacturing of beers from grains: malting, mashing, fermentation, maturation and finishing (Wood, 1998). The potential for mycotoxins or their residues to occur in busaa depends upon the initial cereal and the malt (Nkwe et al., 2005; Mbugua and Gathumbi, 2004; Mbugua and Mwaura, 1996), and the fate of the mycotoxin during malting, brewing and fermentation (Jacobsen, 2008; Wood, 1998). Previous studies have reported high incidences and levels of mycotoxins in lager beers and traditional opaque beers (Nkwe et al., 2005; Mbugua and Gathumbi, 2004; Kenji et al., 2000). This paper reports the incidences and levels of aflatoxin, fumonisin and deoxynivalenol in the Kenyan traditional brew, busaa.

MATERIALS AND METHODS

Study site

The study was carried out in the north-eastern part of Bomet East Constituency in Bomet county, Kenya. Agriculture is highly relied upon as the main source of income in the region but some families depend on brewing as an additional source of income.

Sampling design

Busaa samples were collected from consenting brewers in the north-eastern part of Bomet county. Upon obtaining an informed consent from brewers, approximately 100 ml of busaa samples were bought and aliquoted into two 50 ml sterile falcon tubes and transported to the Kenya Medical Research Institute at 2 to 8°C for mycotoxin analysis. A total of 61 samples of busaa were collected and investigated. The busaa samples were stored at -40°C prior to analysis.

Extraction and quantification of mycotoxins

The samples were first allowed to thaw to room temperature (16 to 26°C) prior to analysis. Aflatoxin, fumonisin and deoxynivalenol were detected and quantified using the Envirologix QuickTox Kit for QuickScan according to the manufacturer's instructions. The EnviroLogix QuickTox kits for QuickScan fumonisin and deoxynivalenol can detect the respective residues ranging from 0.20 to 6.0 ppm, while the EnviroLogix QuickTox kits for QuickScan aflatoxin can detect aflatoxin residues ranging from 2.5 to 30 ppb. Each sample was first vortexed for 15 s prior to mycotoxin extraction. To extract aflatoxin, 5 ml of busaa was measured into a disposable sample cup. 10 ml of ethanol was added and placed on a shaker for 2 min to extract aflatoxin. The mixture was then allowed to separate into two layers. 100 µl of the top yellowish layer was diluted by addition of 100 µl of DB2 buffer in reaction vials. The buffer and the sample extracts were mixed thoroughly by drawing up and down in the pipette tip until the mixtures were uniformly yellow. Aflatoxin QuickTox Strips were placed into the reaction vials and allowed to develop for 5 min. The bottom section of each strip covered by the arrow tape was immediately cut off and discarded while the strips were inserted into the QuickScan reader for quantification.

To extract fumonisin, 5 ml of busaa were measured into a disposable sample cup and 10 ml of ethanol was added and placed on a shaker 2 min to extract fumonisin. 100 µl of the top yellowish layer of the extract was diluted with 100 µl of 50% ethanol in a dilution vial. Mixing was done by drawing up and down in the pipette tip until the mixture was uniformly yellow. 100 µl of the diluted extract was mixed with 100 µl of DB2 Buffer in a second reaction vial. Fumonisin QuickTox strips were placed in the mixtures and allowed to develop for 5 min. The bottom section of each strip covered by the arrow tape was immediately cut off and discarded then the strip was inserted into the QuickScan reader for quantification. To extract DON, 5 ml of busaa was measured into a disposable sample cup and 25 ml of room temperature tap water was added then placed on a shaker for 30 s. The extract was allowed to separate into two layers and the top tan layer was used in the test. 100 µl of the top tan layer was mixed with 100 µl of DB1 buffer in a reaction vial. DON QuickTox strips were placed in the reaction vials and allowed to develop for 10 min. The bottom section of each strip covered by the arrow tape was immediately cut off and discarded and the strip was inserted into the QuickScan reader for quantification.

RESULTS AND DISCUSSION

This study shows that the incidence of the mycotoxins tested were as follows: aflatoxin 93% (mean: 5.2±0.2 µg/kg; range 2.8 to 11 µg/kg), fumonisin 9.8% (mean: 1460±188 µg/kg; range 280 to 4000 µg/kg) and DON 23% (mean: 259±5.2 µg/kg; range: 200 to 360 µg/kg). 65.6% of the samples had aflatoxin levels above the limit set by the European Union (4 µg/kg) (Table 1). Co-occurrence of the mycotoxins was also observed in the samples. Aflatoxin and fumonisin co-occurred in 9.8% of the samples, aflatoxin and DON co-occurred in 23% while all the mycotoxins co-occurred in 3.3% of the

Table 1. The incidence of aflatoxin, fumonisin and DON in busaa from Bomet County, Kenya in 2011.

Mycotoxin	Number of sample assayed	Number of samples above LOD	Incidence (%)	Mean (µg/kg)	Range (µg/kg)
Aflatoxin	61	57[1]	93	5.2 ± 0.2[1]	2.8 - 11
Fumonisin	61	6[2]	9.8	1460 ± 188[2]	280 - 4000
DON	61	14[3]	23	259 ± 5.2[3]	200 - 360

[1] LOD = 2.5 µg/kg, [2] LOD = 200 µg/kg, [3] LOD = 200 µg/kg.

samples.

This study was carried out in a rural setup where agriculture is the main economic activity and brewing is practiced by some women as an additional source of income. The ingredients of the brew are usually obtained locally or from one's own produce. However, the study was carried out at a time when the region was facing maize shortage due to attack by maize lethal necrotic disease hence most brewers had temporarily stopped brewing. Additionally, most maize available for sale had been brought into the county from other regions mostly from the North Rift region of Kenya. Busaa is a product of a two-stage fermentation process. In the first stage, water is added to raw maize flour to form a stiff mixture then covered and allowed to ferment at ambient temperatures 22 to 30°C for 2 to 3 days. The acidified mixture is then roasted, usually on a large metal sheet, resulting in a desirable roasted flavor. The roasted product is mixed with water and finely ground finger millet malt is added and allowed to ferment for another 2 to 3 days. This second fermentation phase results in production of lactic acid and alcohol. Good quality busaa is opaque and creamy brown at the time of consumption due to consistent dispersion of starch and other cereal residues. Busaa should be consumed as soon as it is ready for consumption because prolonged storage results in fermentative acidification which leads to loss of consistent dispersion of starch and separation of the sediment. Due to the visual deterioration and increased sourness, the product becomes unacceptable and is usually rejected by its consumers. Therefore, there is usually a mutual agreement among brewers and consumers of busaa to first exhaust a drink from one brewer before proceeding to the next. Brewers also brew in turns so as to minimize spoilage.

This whole process of busaa production involves ingredients, moist conditions and ambient temperatures that favor fungal growth and hence mycotoxin production. Maize, the main ingredient of busaa, has been found to be an excellent substrate for mycotoxin production given a favorable environment for mycotoxigenic fungal growth (Alakonya et al., 2009). Kenji et al. (2000) reported as high as 1120 µg/kg of total aflatoxins in malted maize and 86% incidence of aflatoxin B1. Besides, the malting process of finger millet and the fermentation of the maize flour, the dough allow the contaminating fungi to grow and produce mycotoxins especially due to the poor hygienic handling conditions involved. Kenji (2003) studied aflatoxins in busaa in the slums of Nairobi and found out that 68% of the samples analyzed had concentrations of aflatoxin higher than 5 ppb and 17% were above 50 ppb. Nkwe et al. (2005) however detected neither aflatoxin nor fumonisin in sorghum-based traditional malt in Botswana. Deoxynivalenol has been reported to be a heat stable toxin that can be found in cereals such as wheat and maize as well as their products (Jacobsen, 2008). Although there is a roasting step during busaa preparation, the mycotoxins still persist due to their stability. Mbugua and Gathumbi (2004) also reported the presence of fumonisins and DON in Kenyan Pilsner and Tusker beers. While the incidence of fumonisin and DON were low in this study, as well as their levels being within the limits set by the European Union (400 and 1750 µg/kg, respectively), the effects of the toxins are still a cause of concern if the volumes of busaa consumed daily are to be considered. It has been reported that consumers of busaa can drink as much as 2 L per day per person almost on a daily basis (Kenji, 2003).

The co-occurrence of multiple mycotoxins even in low quantities may have higher toxicity with serious health consequences for regular busaa consumers. Although, exposure to low levels of mycotoxins may not cause immediate effects, exposure to these toxins over a long period of time may result in long term effects. This may be attributed to the high incidence of esophageal cancer in Bomet County as reported by the findings of Wakhisi et al. (2005). Additionally, since it is a common practice in the Kenyan rural areas for busaa consumers to exhaust a drink from one brewer before proceeding to the next, there is obvious exposure to different mycotoxins occurring at different levels daily. The possibility of passage of mycotoxins from the raw materials to the brew should not be ignored as spoilt maize grains are often diverted to traditional brewing or animal feeds. The purity of the ingredients of traditional brews determines the purity of the brew as suggested by the findings of Nkwe et al. (2005)

CONCLUSION AND RECOMMENDATION

This study confirms the presence of multiple mycotoxins in busaa from Bomet county which has the highest rate of

esophageal cancer in the world. Regular consumption of the traditional brew therefore poses a health risk to its consumers. The study was carried out at a time that the region was facing maize shortage and for more satisfactory findings of mycotoxin contamination in busaa, we recommend further studies when there is a bumper harvest. We also recommend mycotoxicological quality control of traditionally produced brew so as to ensure public mycotoxicological safety.

Conflict of Interests

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

REFERENCES

Ajeyuyo OO, Olowu RA, Agbaje TO, Atamenwan M, Osundiya MO (2011). Enzyme-Linked Immunosorbent Assay (ELISA) of Aflatoxin B1 in Groundnut and Cereal Grains in Lagos, Nigeria. Res. J. Chem. Sci. 1(8):1-5.

Alakonya AE, Monda EO Ajanga S (2009). Fumonisin B_1 and Aflatoxin B_1 levels in Kenyan maize. J. Plant Pathol. 91(2):459-464.

Bennett JW, Klich M (2003). Mycotoxins. Clin. Microbiol. Rev. 16:497–516.

Binder EM, Tan LM, Chin LJ, Handl J, Richard J (2007).Worldwide occurrence of mycotoxins in commodities, animal feed and feed ingredients. Anim. Feed Sci. Technol. 137:265-282.

Daško Ĺ, Rauová D, Belajová E, Kováć M (2005). Determination of Fumonisin B1 and B2 in Beer. Czech. J. Food Sci. 23:20-26.

Jacobsen BJ (2008). Predicting the Incidence of Mycotoxins. In: 26th Annual International Symposium on Man and His Environment in Health and Disease. Conference Proceedings, June 19-20, 2008, USA.

Kedera CJ, Plattner RD, Desjardins AE (1998). Incidence of *Fusarium spp*. and levels of fumonisin B_1 in maize in western Kenya. Appl. Environ. Microbiol. 65(1):41-44.

Kenji GM, Mvula AM, Koaze H, Baba N (2000). Aflatoxin contamination of Kenyan maize flour and malted Kenyan and Malawian Grains. Sci. Report Facul. Agric. Okayama University 89:5-7.

Kenji G (2003). Aflatoxins in traditional beer (Busaa) in Nairobi, Kenya. AJFAND 1684-5378.

Kuiper-Goodman T (2002). Recent developments in the risk assessment of deoxynivalenol. Toxicology, quality and impact on industry, Second Canadian workshop on *Fusarium* head blight, Ottawa. Dostupan: http://res2.agr.gc.ca/ecorc/fusarium01/session2.pdf

Mbugua SK, Mwaura SM (1996). Aflatoxin contamination in fermented and germinated products in Kenya. Proceedings of 3rd Biennial Seminar on African fermented foods, Accra.

Mbugua SK, Gathumbi JK (2004). The contamination of Kenyan lager beers with *Fusarium* mycotoxins. J. Inst. Brew. 110(3):227-229.

Milićević DR, Škrinjar M, Baltić T (2010). Real and Perceived Risks for Mycotoxin Contamination in Food and Feeds: Challenges for Food Safety Control. Toxins 2:572-592.

Moturi WKN (2008). Factors likely to enhance mycotoxin introduction into the human diet through maize in Kenya. AJFAND 8(3):265-277.

Nkwe JE, Taylor JE Siame BA (2005). Fungi, aflatoxins, fumonisin BI and zearalenone contaminating sorghum-based traditional malt, wort and beer in Botswana. Mycopathologia 160**:**177-186.

Paul KC, Nceba G, Michael FD, Anil AC (2001). Exposure of Rural and Urban Population in Kwazulu Natal, South Africa to Fumonisin B in Maize. Environ. Health Perspect. 109:253-260.

Shephard GS (2008). Risk assessment of aflatoxins in food in Africa. Food Addit. Contam. 25(10):1246-1256.

Sudakin DL (2003). Trichothecenes in the environment: relevance to human health. Toxicol. Lett. 143:97-107.

Wakhisi J, Patel K, Buziba N, Rotich J (2005). Esophageal cancer in north rift valley of western Kenya. Afr. Health Sci. 5(2):157-163.

Wood BJB (1998). Microbiology of Fermented Foods. 2nd ed. Netherlands: Chapman and Hall.

academicJournals

Vol. 13(26), pp. 2679-2688, 25 June, 2014
DOI: 10.5897/AJB2014.13815
Article Number: E0695DF45614
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Hepatoprotective effects of *Allium cepa* (onion) extracts against paracetamol-induced liver damage in rats

Ozougwu, J. C.[1]* and Eyo, J. E.[2]

[1]Physiology and Biomedical Research Unit, Department of Biological Sciences, Rhema University, Aba, Abia State, Nigeria.
[2]Physiology and Biomedical Research Unit, Department of Zoology and Environmental Biology, University of Nigeria, Nsukka, Nigeria.

Received 25 March, 2013; Accepted 16 June, 2014

Liver damage due to paracetamol hepatotoxicity is a major health challenge worldwide. It is against this background that this study was designed to determine the hepatoprotective effects of the increasing dosage of *Allium cepa* methanolic extracts on paracetamol induced hepatotoxic rats. Fifty-four (54) adult male albino rats comprising of nine normal and 45 paracetamol hepatotoxic rats were used for this study. The experimental design was the three by three Latin square design. Paracetamol hepatotoxicity was induced by single administration of paracetamol at 750 mg/kg ip on the first day of the experiment. The different biochemical parameters assessed were determined before the start of the study and subsequently monthly for the duration of the study. Blood samples were collected from the rat through the eye monthly for analysis and serum was obtained by centrifugation (5000 rpm for 10 min) and stored at -20°C prior to analysis. The effects of duration and increasing dosages (200, 300 and 450 mg/kg) of *A. cepa* methanolic extracts produced a duration dependent significant ($p < 0.05$) reductions in the alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and total serum bilirubin (TSB) of paracetamol hepatotoxic rats after the duration of study when compared with those of the paracetamol, normal and silymarin control rats. *A. cepa* reduced alanine aminotransferase and total serum bilirubin in a dose dependent fashion whereas it reduced aspartate aminotransferase, alkaline phosphatase and lactate dehydrogenase level in a dose independent manner. *A. cepa* extracts studied showed potent hepatoprotective properties. It was evident that *A. cepa* extracts was able to reduce significantly all the elevated biochemical parameters due to paracetamol hepatotoxicity and this was collaborated by results of histopathological studies.

Key words: *Allium cepa,* paracetamol, hepatoprotective effects, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase, total serum bilirubin.

INTRODUCTION

The liver is one of the most vital organs that functions as a centre for metabolism of nutrients and excretion of waste metabolites. The liver handles the metabolism and excretion of drugs from the body thereby providing protection against foreign substances by detoxifying and eliminating them (Mohamed-Saleem et al., 2010). Given

the liver's strategic function in the body, it is continuously and variedly exposed to xenobiotics, environmental pollutants and chemotherapeutic agents (Gupta and Misra, 2006). Hepatic dysfunction due to paracetamol overdose is increasing worldwide and medicinal plants are a major constituent of various alternative systems of medicines used worldwide since ancient times. To rationalize the use of herbal preparations in the management of hepatotoxicity, a scientific research on them is needed. This is moreso as therapies developed along the principles of western medicine often carry the risk of adverse effects and are often too costly especially for the developing countries of the world. *Allium cepa* (onion) is a bulbous plant widely cultivated with leading production in China, India and United states. It is rich in proteins, carbohydrates, sodium, potassium and phosphorus. Traditionally, onion has been used to treat intestinal infections (Shaik et al., 2012). It has been reported to be an antibacterial, antiviral, antiparasitic, antifungal and has antihypertensive, hypoglycemic, antithrombotic, anti-hyperlipidemic, anti-inflammatory and antioxidant activity (Abdul et al., 2010; Ozougwu et al., 2008; Eyo et al*.,* 2011; Ozougwu, 2011). This present study was designed to determine the hepatoprotective effects of increasing dosage of *A. cepa* (onions) methanolic extracts against paracetamol induced liver damage in rats viz a viz biochemical parameters such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and total serum bilirubin (TSB) level of paracetamol induced hepatotoxic rats.

MATERIALS AND METHODS

Plant materials

The *A. cepa* used for the study was bought from the Ogige market, Nsukka, Enugu state, Nigeria. The plants were identified (Gbile, 1980) to species level at the Herbarium Unit, Department of Plant Science and Biotechnology, University of Nigeria, Nsukka.

Animal model

Fifty- four (54) adult white wistar strain male albino rats (*Rattus norvegicus*) weighing 180 to 200 g were used for the study. They were fed *ad labitum* with 18% crude protein (Guinea feed) commercial feed and allowed to acclimatize for two weeks under standard photoperiodic condition in a clean rat cage with three rats per cage in the research laboratory. All animals were maintained under the standard laboratory condition for temperature (26 ± 2°C), humidity (50 ± 5%) and light (12 h day length) and were allowed free access to food and water.

Preparation of plant extracts

Fresh healthy *A. cepa* bulbs were washed, cut into small pieces and homogenized in a warring blender. The resulting mixture was soaked in 2 L of 80% methanol. The mixture was allowed to stand for 24 h with intermittent shaking. Following filtration, the filtrate obtained was concentrated to dryness at 40°C using a rotary evaporator under reduced pressure. The dried extracts were weighed and then stored in a refrigerator.

Induction of paracetamol hepatotoxicity in rats

The minimum dose of paracetamol that causes death in rats is 1060 mg/kg and the median lethal dose (LD_{50}) is 765 mg/kg (Boyd and Hogan, 1968). Paracetamol hepatotoxicity was induced by single administration of solution of paracetamol at 750 mg/kg intraperitoneally. After 4 days only rats with ALT levels above 65 U/l were considered as hepatotoxic and used for the study. Several researchers have induced hepatotoxicity in rats by single administration of solution of paracetamol on rats at 750 mg/kg ip (Hamid et al., 2011; Reddy et al., 2012; Rafi et al., 2013).

Experimental design

The study was carried out on paracetamol- induced hepatotoxic rats for 12 weeks. The experimental design was the three by three Latin square design. Fifty-four rats used were divided into two major groups: Group I, Nine non-hepatotoxic rats (Normal control); Group II, Forty-five paracetamol induced hepatotoxic rats.

The group I rats were three rats each in three different cages and each received 1 ml/kg of 5% methanol solution daily throughtout the duration of the study. The Group II rats (paracetamol induced hepatotoxic rats) were divided into three subgroups (IIa, IIb, IIc). The subgroup IIa was the paracetamol control, three rats in a cage, and was replicated thrice and had 3 rats each which received 750 mg/kg of paracetamol only (Sumy et al., 2011; Iqbal et al., 2007). Subgroup IIb was divided into 3 replicates (IIb1, IIb2, and IIb3) respectively each replicate had 3 rats and received 200, 300 or 450 mg/kg of *A. cepa* methanolic extracts orally daily. The subgroup IIc, three rats each in a cage, and replicated thrice received the standard drug silymarin at 100 mg/kg (Yuvaraj and Subramoniam, 2009). The different biochemical parameters (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase and total serum bilirubin) assessed were determined first before the start of the experiment and subsequently monthly for the duration of the study. Blood samples were collected from the rat through the eye monthly for analysis. Serum was obtained by centrifugation (5000 rpm for 10 min) and stored at -20°C prior to analysis.

Evaluation of biochemical parameters

Serum alanine aminotransferase and aspartate aminotransferase levels were determined by colorimetric method of Reitman and Frankel (1957) and absorbance was read at 505 nm using spectrophotometer. Alkaline phosphatase level in serum was

*Corresponding author. E-mail: jevaschubby@yahoo.com. Tel: +2348034006816.

Abbreviations: ALT, Alanine aminotransferase; **AST,** aspartate aminotransferase; **ALP,** alkaline phosphatise; **LDH,** lactate dehydrogenase; **TSB,** total serum bilirubin.

Table 1. Effects of the increasing dosage of *A. cepa* extracts on alanine aminotransferase level of paracetamol induced hepatotoxic rats.

Treatments	Dosage	Alanine aminotransferase level (U/L)				% change after the study
		Week 0	Week 4	Week 8	Week 12	
ME	1.0 ml/kg	$49.70 \pm 3.27^{1,a}$	$48.77 \pm 3.10^{1,a}$	$51.00 \pm 2.52^{1,a}$	$50.63 \pm 3.30^{1,a}$	-
PARA	750 mg/kg	$49.27 \pm 3.15^{1,a}$	$72.41 \pm 4.00^{2,b}$	$75.36 \pm 4.52^{3,2,b}$	$78.34 \pm 5.40^{4,b}$	8.19
AC	200 mg/kg	$49.31 \pm 2.66^{1,a}$	$63.33 \pm 2.67^{2,c}$	$60.67 \pm 3.41^{3,2,c}$	$60.98 \pm 2.31^{4,2,c}$	-15.79
AC	300 mg/kg	$49.31 \pm 2.76^{1,a}$	$64.82 \pm 2.16^{2,d,c}$	$58.33 \pm 2.33^{3,d,c}$	$57.44 \pm 2.40^{4,3,d}$	-20.67
AC	450 mg/kg	$48.73 \pm 3.12^{1,a}$	$68.48 \pm 4.67^{2,e}$	$56.74 \pm 2.57^{3,e,d}$	$56.49 \pm 2.56^{4,3,e,d}$	-21.99
SL	100 mg/kg	$50.57 \pm 3.83^{1,a}$	$52.41 \pm 3.621^{l,g,j}$	$51.94 \pm 2.18^{1,a}$	$50.58 \pm 2.56^{1,a}$	-30.15

Values given represents the Mean ± SD of 9 observations, mean values labeled with the same number superscripts along the same row are not significantly different at 5% significance level ($p < 0.05$). Mean values labeled with the same alphabets superscripts on the same column are not significantly different at 5% significance level ($p < 0.05$). ME = 5% Methanol solution representing the normal control; PARA = Paracetamol control; AC = *Allium cepa* ; SL= Silymarin representing standard drug. % change = % change from para treated at weeks 4 compared to all treatments groups at week 12. Negative % change denotes decrease, Positive % change denotes an increase.

determined by the method of King and King (1954). Serum was incubated with disodium phenylphosphate as substrate buffered at pH 10 for 15 min at 37°C. The hydrolytic products, phenol was condensed with 4-amino antipyrine and then oxidized with alkaline ferrcyanide and the red complex developed was read at 510 nm using spectrophotometer. Lactate dehydrogenase level was estimated by the method of Babson and Babson (1973), where the reduction of nucleoside derived amino acids (NAD) was coupled with the reduction of tetrazolium salt and the produced formazan was measured using spectrophotometer at 503 nm. Total serum bilirubin was determined following the method of Mallory and Evelyn (1937). Diazotised sulphonilic acid reacts with bilirubin in diluted serum and forms purple colored azobilirubin which was read at 540 nm using spectrophotometer.

Data analysis

The data collected was pooled and analyzed for their central tendencies using descriptive statistic, values were given as mean ± standard deviation of the observations. Analysis of variance and LSD was employed to test the significant differences ($P < 0.05$) among treatment means. All analyses were performed using SPSS for windows statistical software package version 16. The resulting outputs were presented in tables.

RESULTS

Alanine aminotransferase level

The increasing dosages (200, 300 and 450 mg/kg) of *A. cepa* methanolic extracts produced a duration dependent significant ($p < 0.05$) reductions in the alanine amino-transferase levels of paracetamol hepatotoxic rats after the duration of treatment when compared with those of the paracetamol and silymarin control rats. Alanine aminotransferase levels were significantly higher in paracetamol control groups throughout the duration of the study compared to all other treatment groups whereas it was significantly higher in all groups at the same period compared to the normal group (1 ml/kg of 5% methanol solution). *A. cepa* reduced alanine aminotransferase level in a dose dependent fashion across the duration of the study with *A. cepa* at 200 mg/kg reducing alanine aminotransferase level by 15.79%, at 300 mg/kg it was reduced by 20.67% whereas at 450 mg/kg it was lowered by 21.99% after the duration of treatments when com-pared to paracetamol control at week 4. Silymarin reduced alanine aminotransferase level by 30.15% after the duration of treatment compared with paracetamol control at week 4 (Table 1). Normal control had no significant effect on alanine aminotransferase level whereas the paracetamol treated control raised alanine aminotransferase level by 8.19%.

Aspartate aminotransferase level

The increasing dosages (200, 300 and 450 mg/kg) of *A. cepa* methanolic extracts produced a duration dependent significant ($p < 0.05$) reductions in the aspartate aminotransferase level of paracetamol hepatotoxic rats after the duration of treatment when compared with those of the paracetamol and silymarin control rats. Aspartate aminotransferase level were significantly higher in parace-tamol control groups throughout the duration of the study compared to all other treatment groups whereas it was significantly higher in all groups at the same period compared to the normal group. *A. cepa* reduced aspartate aminotransferase level in a dose independent fashion across the duration of study with *A. cepa* at 200 mg/kg reducing aspartate aminotransferase level by 41.77% at 300 mg/kg it was reduced by 39.57% whereas at 450 mg/kg it was lowered by 38.59% after the duration of the study when compared to paracetamol control at week 4. Silymarin at 100 mg/kg reduced aspartate aminotransferase level by 62.26% after the duration of treatment compared with paracetamol control at week 4 (Table 2). Normal control had no significant effect on aspartate aminotransferase level whereas the parace-tamol treated control raised aspartate amino-transferase level by 8.97%.

Table 2. Effects of the increasing dosage of *A. cepa* extracts on aspartate aminotransferase level of paracetamol - induced hepatotoxic rats.

Treatments	Dosage	Aspartate aminotransferase level (U/L)				% change after the study
		Week 0	Week 4	Week 8	Week 12	
ME	1.0 ml/kg	$86.76 \pm 2.84^{1,a}$	$86.62 \pm 2.49^{1,a}$	$86.32 \pm 2.68^{1,a}$	$87.10 \pm 2.58^{1,a}$	-
PARA	750 mg/kg	$87.17 \pm 2.41^{1,a}$	$659.97 \pm 12.00^{2,b}$	$688.42 \pm 20.83^{3,b}$	$725.01 \pm 12.09^{4,b}$	8.97
AC	200 mg/kg	$87.09 \pm 2.44^{1,a}$	$451.21 \pm 10.53^{2,c}$	$418.89 \pm 11.77^{3,c,d,e}$	$384.30 \pm 18.95^{4,c}$	- 41.77
AC	300 mg/kg	$86.89 \pm 2.81^{1,a}$	$430.18 \pm 8.39^{2,d}$	$421.72 \pm 9.28^{2,d,e}$	$398.80 \pm 13.28^{3,d,e}$	-39.57
AC	450 mg/kg	$86.64 \pm 2.76^{1,a}$	$460.36 \pm 13.75^{2,e}$	$412.47 \pm 12.46^{3,e}$	$405.31 \pm 13.79^{3,e}$	-38.59
SL	100 mg/kg	$86.53 \pm 2.63^{1,a}$	$278.68 \pm 17.63^{2,l}$	$257.12 \pm 10.03^{3,l}$	$249.09 \pm 7.64^{3,l}$	-62.26

Values given represents the Mean ± SD of 9 observations, mean values labeled with the same number superscripts along the same row are not significantly different at 5% significance level ($p < 0.05$). Mean values labeled with the same alphabets superscripts on the same column are not significantly different at 5% significance level ($p < 0.05$). ME = 5% Methanol solution representing the normal control; PARA = Paracetamol control; AC = *Allium cepa* ; SL= Silymarin representing standard drug. % change = % change from para treated at weeks 4 compared to all treatments groups at week 12. Negative % change denotes decrease, Positive % change denotes an increase.

Table 3. Effects of the increasing dosage of *A. cepa* extracts on alkaline phosphatase level of paracetamol induced hepatotoxic rats

Treatment	Dosage	Alkaline phosphatase level (U/L)				% change after the study
		Week 0	Week 4	Week 8	Week 12	
ME	1.0 ml/kg	$112.44 \pm 1.91^{1,a}$	$112.64 \pm 1.86^{1,a}$	$112.07 \pm 1.62^{1,a}$	$112.40 \pm 1.72^{1,a}$	-
PARA	750 mg/kg	$112.16 \pm 2.02^{1,a}$	$904.34 \pm 13.31^{2,b}$	$941.61 \pm 13.83^{3,b}$	$958.60 \pm 19.67^{4,b}$	6.00
AC	200 mg/kg	$112.99 \pm 1.93^{1,a}$	$449.06 \pm 17.85^{2,c}$	$440.41 \pm 26.60^{3,2,c}$	$425.62 \pm 17.63^{4,c}$	-52.94
AC	300 mg/kg	$112.07 \pm 2.15^{1,a}$	$418.96 \pm 10.28^{2,d}$	$417.40 \pm 10.57^{3,2,d}$	$418.12 \pm 12.37^{4,2,d,c}$	-53.77
AC	450 mg/kg	$112.19 \pm 2.28^{1,a}$	$460.24 \pm 9.86^{2,e}$	$446.08 \pm 8.72^{3,e,c}$	$446.08 \pm 8.72^{4,3,e}$	-50.68
SL	100 mg/kg	$112.33 \pm 1.80^{1,a}$	$249.69 \pm 15.30^{2,l}$	$249.69 \pm 15.29^{3,2,l}$	$219.78 \pm 10.25^{4,l}$	-75.70

Values given represents the Mean ± SD of 9 observations, mean values labeled with the same number superscripts along the same row are not significantly different at 5% significance level ($p < 0.05$). Mean values labeled with the same alphabets superscripts on the same column are not significantly different at 5% significance level ($p < 0.05$). ME = 5% Methanol solution representing the normal control; PARA = Paracetamol control; AC = *Allium cepa* ; SL= Silymarin representing standard drug. % change = % change from para treated at weeks 4 compared to all treatments groups at week 12. Negative % change denotes decrease, Positive % change denotes an increase.

Alkaline phosphatase level

The increasing dosages (200, 300 and 450 mg/kg) of *A. cepa* methanolic extracts produced a duration dependent significant ($p < 0.05$) reductions in the alkaline phosphatase level of paracetamol hepatotoxic rats after the duration of treatment when compared with those of the paracetamol and silymarin control rats. Alkaline phosphatase level were significantly higher in paracetamol control groups throughout the duration of the study compared to all other treatment groups whereas it was significantly higher in all groups at the same period compared to the normal group. *A. cepa* reduced alkaline phosphatase level in a dose independent manner with *A. cepa* at 200 mg/kg reducing alkaline phosphatase level by 52.94%, at 300 mg/kg it was reduced by 53.77% whereas at 450 mg/kg it was lowered by 50.68% after the duration of the study when compared to paracetamol control at week 4. Silymarin at 100 mg/kg reduced alkaline phosphatase level by 75.70% after the duration of treatment compared with paracetamol control at week 4 (Table 3). Normal control had no significant effect on alkaline phosphatase level whereas paracetamol treated control raised alkaline phosphatase level by 6.00%.

Lactate dehydrogenase level

The increasing dosages (200, 300 and 450 mg/kg) of *A. cepa* methanolic extracts produced a duration dependent significant ($p < 0.05$) reductions in the lactate dehydrogenase level of paracetamol hepatotoxic rats after the duration of the study when compared with those of the paracetamol and silymarin control rats. Lactate dehydrogenase levels were significantly higher in paracetamol control groups throughout the duration of the study compared to all other treatment groups whereas it was significantly higher in all groups at the same period compared to the normal group. *A. cepa* reduced lactate dehydrogenase level in a dose independent manner with *A. cepa* at 200 mg/kg reducing lactate dehydrogenase level by 42.16%, at 300 mg/kg it was reduced by 44.37% whereas at 450 mg/kg it was lowered by 43.61% after the duration of the study when compared to paracetamol control at week 4. Silymarin at 100 mg/kg reduced lactate dehydrogenase level by 63.08% after the duration of the

Table 4. Effects of the increasing dosage of *A. cepa* extracts on lactate dehydrogenase level of paracetamol induced hepatotoxic rats

Treatments	Dosage	Lactate dehydrogenase level (IU/L)				% change after the study
		Week 0	Week 4	Week 8	Week 12	
ME	1.0 ml/kg	$103.78 \pm 5.54^{1,a}$	$105.00 \pm 5.15^{1,a}$	$104.56 \pm 5.20^{1,a}$	$103.44 \pm 5.13^{1,a}$	-
PARA	750 mg/kg	$104.67 \pm 5.57^{1,a}$	$390.89 \pm 18.21^{2,b}$	$409.67 \pm 10.71^{3,b}$	$417.44 \pm 9.22^{4,b}$	6.79
AC	200 mg/kg	$103.11 \pm 4.96^{1,a}$	$250.22 \pm 9.50^{2,c}$	$234.33 \pm 10.26^{3,c}$	$226.11 \pm 10.26^{4,c}$	-42.16
AC	300 mg/kg	$104.22 \pm 6.04^{1,a}$	$267.33 \pm 13.64^{2,d}$	$248.00 \pm 14.44^{3,d}$	$217.44 \pm 11.81^{4,d,c}$	-44.37
AC	450 mg/kg	$105.67 \pm 5.43^{1,a}$	$105.67 \pm 5.42^{1,a}$	$261.44 \pm 9.32^{2,e}$	$220.44 \pm 9.36^{3,e,d,c,b}$	-43.61
SL	100 mg/kg	$103.22 \pm 5.26^{1,a}$	$165.11 \pm 18.15^{2,j}$	$165.11 \pm 18.15^{3,2,l}$	$144.33 \pm 13.10^{4,l}$	-63.08

Values given represents the Mean ± SD of 9 observations, mean values labeled with the same number superscripts along the same row are not significantly different at 5% significance level ($p < 0.05$). Mean values labeled with the same alphabets superscripts on the same column are not significantly different at 5% significance level ($p < 0.05$). ME = 5% Methanol solution representing the normal control; PARA = Paracetamol control; AC = *Allium cepa* ; SL= Silymarin representing standard drug. % change = % change from para treated at weeks 4 compared to all treatments groups at week 12. Negative % change denotes decrease, Positive % change denotes an increase.

Table 5. Effects of the increasing dosage of *A. cepa* extracts on total serum bilirubin levels of paracetamol induced hepatotoxic rats.

Treatments	Dosage	Total serum bilirubin level (mg/dl)				% change after the study
		Week 0	Week 4	Week 8	Week 12	
ME	1.0 ml/kg	$0.52 \pm 0.16^{1,a}$	$0.53 \pm 0.15^{1,a}$	$0.54 \pm 0.16^{1,a}$	$0.51 \pm 0.13^{1,a}$	-
PARA	750 mg/kg	$0.55 \pm 0.15^{1,a}$	$3.57 \pm 0.37^{2,b}$	$4.19 \pm 0.36^{3,b}$	$4.78 \pm 0.49^{4,b}$	33.89
AC	200 mg/kg	$0.53 \pm 0.14^{1,a}$	$2.78 \pm 0.62^{2,c}$	$2.06 \pm 0.53^{3,2,c}$	$1.64 \pm 0.29^{4,c}$	-54.06
AC	300 mg/kg	$0.52 \pm 0.13^{1,a}$	$1.78 \pm 0.41^{2,d}$	$1.45 \pm 0.48^{3,d}$	$1.24 \pm 0.34^{4,3,d}$	-65.27
AC	450 mg/kg	$0.55 \pm 0.15^{1,a}$	$1.63 \pm 0.27^{2,e,d}$	$1.08 \pm 0.34^{3,e}$	$1.15 \pm 0.45^{4,3,e,d}$	-67.79
SL	100 mg/kg	$0.52 \pm 0.13^{1,a}$	$0.93 \pm 0.28^{2,l}$	$0.75 \pm 0.25^{1,2,a}$	$0.63 \pm 0.2^{4,1,a}$	-82.35

Values given represents the Mean ± SD of 9 observations, mean values labeled with the same number superscripts along the same row are not significantly different at 5% significance level ($p < 0.05$). Mean values labeled with the same alphabets superscripts on the same column are not significantly different at 5% significance level ($p < 0.05$). ME = 5% Methanol solution representing the normal control; PARA = Paracetamol control; AC = *Allium cepa* ; SL= Silymarin representing standard drug. % change = % change from para treated at weeks 4 compared to all treatments groups at week 12. Negative % change denotes decrease, Positive % change denotes an increase.

study compared with paracetamol control at week 4 (Table 4). Normal control had no significant effect on lactate dehydrogenase level whereas paracetamol treated control raised lactate dehydrogenase level by 6.79%.

Total serum bilirubin level

The increasing dosages (200, 300 and 450 mg/kg) of *A. cepa* methanolic extracts produced a duration dependent significant ($p < 0.05$) reductions in the total serum bilirubin level of paracetamol hepatotoxic rats after the duration of treatment when compared with those of paracetamol and silymarin control rats. Total serum bilirubin levels were significantly higher in paracetamol control groups throughout the duration of the study compared to all other treatment groups whereas it was significantly higher in all groups at the same period compared to the normal group. *A. cepa* reduced total serum bilirubin level in a dose dependent manner across the duration of the study with *A. cepa* at 200 mg/kg reducing total serum bilirubin level by 54.06%, at 300 mg/kg it was reduced by 65.27% whereas at 450 mg/kg it was lowered by 67.79% after the duration of treatments when compared to paracetamol control at week 4. Silymarin at 100 mg/kg reduced total serum bilirubin level by 82.35% after the duration of the study compared with paracetamol control at week 4 (Table 5). Normal control had no significant effect on total serum bilirubin level whereas paracetamol treated control raised total serum bilirubin level by 33.89%.

Histological examination of prepared tissue slides

The histological examination of liver sections under a light microscope basically supported the biochemical results. Liver sections from the normal control rats showed normal appearance of hepatic architecture, normal central vein, hepatocyte and normal hepatic sinusoid (Figure 1). Liver sections of paracetamol control rats showed cloudy swelling of fat droplets, very severely degenerated hepatocytes, very severely conjested sinusoids and damaged central vein (Figure 2). Liver section of rats treated with methanolic extracts of *A. cepa*

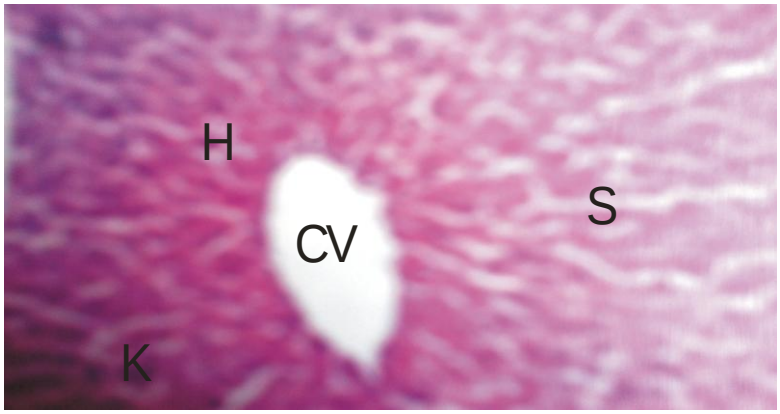


Figure 1. Photomicrograph of transverse section of normal liver treated with 1 ml/kg of 5% methanol solution showing normal appearance of hepatic architecture, normal central vein (CV), hepatocyte (H), Kupffer cells (K) and normal hepatic sinusoid. H & E X 400.

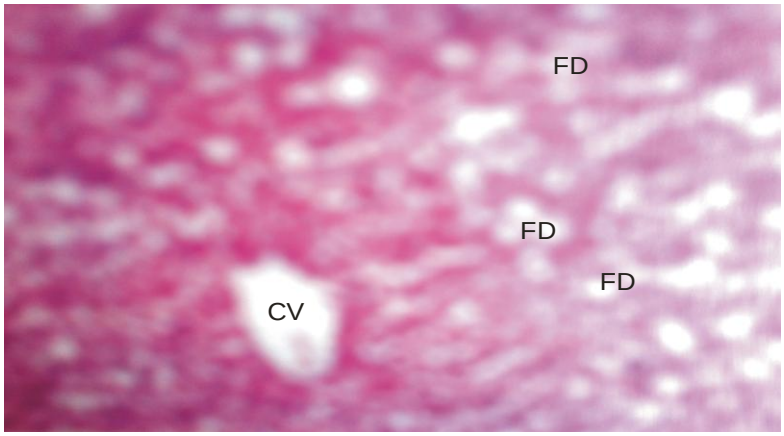


Figure 2. Photomicrograph of transverse section of liver treated with Paractamol at 750 mg/kg showing congested central vein (CV), very severe fat droplets (FD), very severely degenerated hepatocyte (H) and very severely conjested hepatic sinusoid. H & E X 400.

at 200 mg/kg showed distorted hepatic architecture, severely conjested central vein, moderately degenerated hepatocyte and severely congested sinusoid (Figure 3) whereas at 300 mg/kg it showed distorted hepatic architecture, severely congested central vein, moderately degenerated hepatocyte and severely congested sinusoid (Figure 4), at 450 mg/kg it showed severely congested central vein, moderately degenerated hepatocyte and severely congested sinusoid (Figure 5). The liver sections of rats treated with silymarin standard drug at 100 mg/kg showed mildly conjested central vein, moderately degenerated hepatocyte and moderately conjested sinusoid (Figure 6).

DISCUSSION

Paracetamol is a widely used analgesic and antipyretic drug, which is safe in therapeutic doses but can produce fatal hepatic necrosis in man and rats at toxic doses (Mitchell et al*.*, 1973). Paracetamol-induced hepatic injury is commonly used as an experimental model for the study of hepatoprotective effects of medicinal plants and drugs (Plaa and Hewitt, 1982; Gite et al., 2010).

Liver aminotransferases (ALT and AST)

The aminotransferases (ALT and AST) are the most frequently utilized and specific indicators of hepatocellular necrosis (Dama et al*.*, 2011). The significant increase observed in the level of serum aminotransferase (AST and ALT) in paracetamol treated rats compared to the normal rats in this study could be due to hepatocellular damage because these enzymes are normally located in the cytoplasm and released into the circulation after

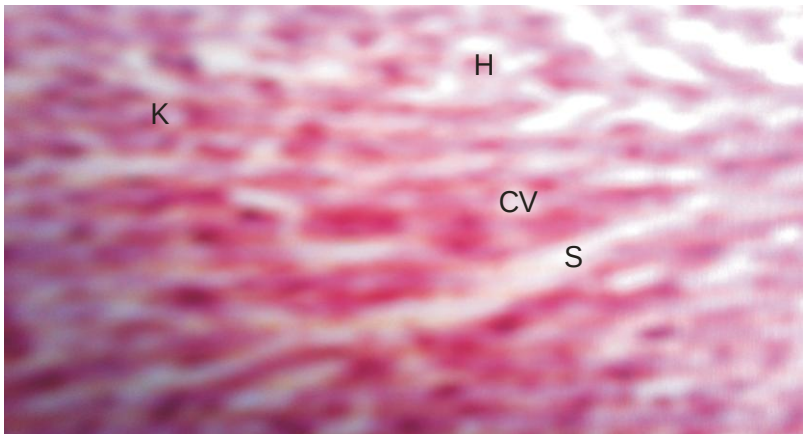


Figure 3. Photomicrograph of transverse section of liver treated with methanolic extracts of *Allium cepa* at 200 mg/kg showing severely congested central vein (CV), moderately degenerated hepatocyte (H), Kupffer cells (K) and severely conjested hepatic sinusoid. H & E X 400.

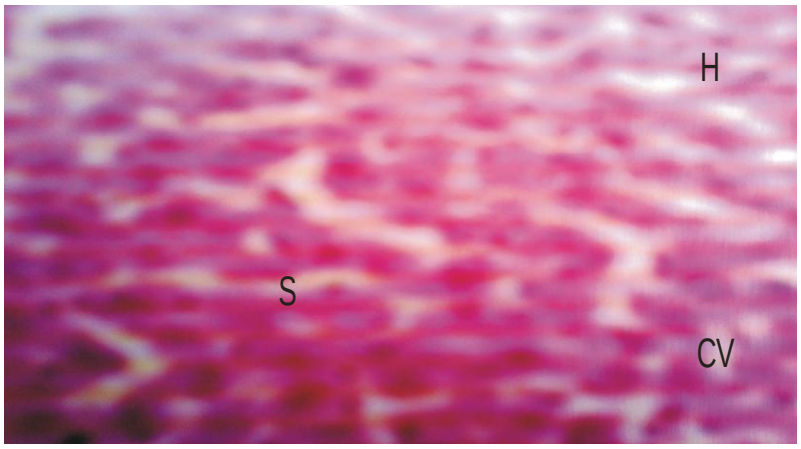


Figure 4. Photomicrograph of transverse section of liver treated with methanolic extracts of *Allium cepa* at 300 mg/kg showing distorted hepatic architecture, severely congested central vein (CV), moderately degenerated hepatocyte (H) and severely conjested sinusoid. H & E X 400.

cellular damage (Hassan and El-Gendy, 2003). The obtained results support the findings of Naziroglu et al. (1999) and Ahmed et al. (2000) who observed similar effects on hepatic enzymes after carbon tetrachloride intoxication in different animals. Administration of silymarin and *A. cepa* on paracetamol treated rats reduced the level of aminotransferase enzymes (AST and ALT) in the serum. The mechanism of action *A. cepa* could be by the prevention of the intracellular enzyme release and its membrane stabilizing and antioxidant effects (Sabina et al., 2011). This is because *A. cepa* are rich in strong antioxidant and are well documented against reactive oxygen species-mediated damage (Ippoushi et al., 2003; Lee et al., 2009). The protective effects of silymarin observed in this study could be attributed to its antioxidant and free radicals-scavenging properties, which has been well established (Horvath et al., 2001).

Alkaline phosphatase (ALP)

In this study the reduction in ALP levels by extracts may suggest repairing of rats liver by *A. cepa* extracts. Possible mechanisms that may be responsible for the protection of paracetamol induced liver damage by *A. cepa* may be by the extract's ability to act as a free radical scavenger intercepting those radicals involved in paracetamol metabolism by microsomal enzymes and also its ability to inhibit rat hepatic microsomal membrane lipid peroxidation. Thus by trapping oxygen related free radicals, *A. cepa* could hinder their interaction with

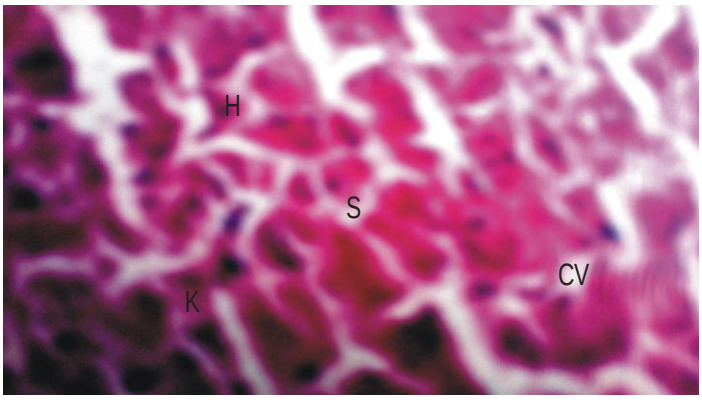


Figure 5. Photomicrograph of transverse section of liver treated with methanolic extracts of *Allium cepa* at 450 mg/kg showing distorted hepatic architecture, severely congested central vein (CV), moderately degenerated hepatocyte (H) and severely conjested sinusoid. H & E X 400.

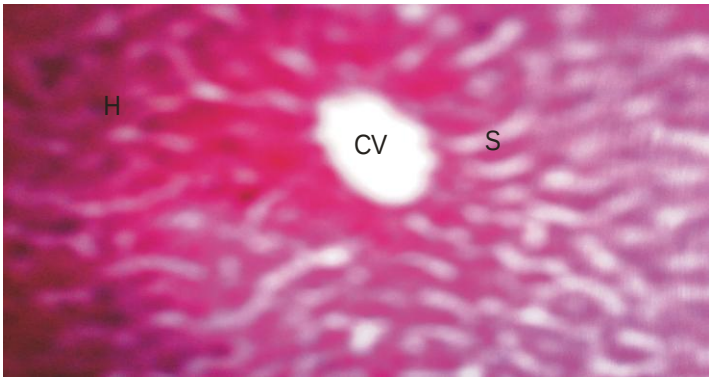


Figure 6. Photomicrograph of transverse section of liver treated with silymarin at 100 mg/kg showing mildly conjested central vein (CV), moderately degenerated hepatocyte (H) and moderately conjested sinusoid (S). H & E X 400.

polyester fatty acids and would abolish the enhancement of lipids peroxidative processes (Aniya et al., 2002; Achuthan et al., 2003; Chattopadhyay, 2003). Another possible mechanism is that the active ingredients in *A. cepa* allyl propyl disulfide could have increased the levels of glutathione which binds to the toxic metabolites of paracetamol such as N- acetyl- p- benzoquinone imine (NAPQI) and increased its rate of excretion from the body. Also, the active ingredients of *A. cepa* extracts might have inhibited the level of the cytochrome P- 450 enzyme system which decreased the formation of NAPQI from ingested paracetamol.

Lactate dehydrogenase level

In this present study, *A. cepa* reduced lactate dehydrogenase level in a dose independent fashion, after the duration of the study. Silymarin reduced lactate dehydrogenase level, paracetamol control raised lactate dehydrogenase level whereas normal control had no significant effect on lactate dehydrogenase level. Rusu et al. (2005) and De-Andrade et al. (2010) reported decrease in hepatic LDH after carbon tetrachloride intoxication but this present study showed increased levels of serum LDH after paracetamol intoxication. The difference could be due to the different hepatotoxin used, dosage, mode of administration and the physiological status of the experimental animals used. The observed increase in LDH in this study could be as a result of enzyme inhibition mainly due to increased membrane fluidity as a result of ROS involvement which led to enzyme leakage into circulation (Hamed, 2011).

Total serum bilirubin

Elevated total serum bilirubin observed in paracetamol

hepatotoxic rats suggested abnormal conjugation of bilirubin by the liver due to generalized hepatocellular damage (El-sherbiny et al., 2003). Total serum bilirubin was decreased in paracetamol hepatotoxic rats after treatment with silymarin and *A. cepa*, this supported the work of Fener et al. (1987) who reported that silymarin is known to have reductive effects on total serum bilirubin in different liver diseases. The possible mechanism of action of *A. cepa* extracts may be through their antioxidative effects. This is because *A. cepa* has active ingredients that are capable of free radical scavenging in living system (Mitra et al., 1998).

Histopathology

The efficacy of any hepatoprotective substance is dependent on its capacity of either reducing the harmful effect or restoring the normal hepatic histology and physiology that has been damaged by a hepatotoxin. Liver sections from the normal control rats showed normal appearance of hepatic architecture, normal central vein, hepatocyte, kupffer cells and normal hepatic sinusoid (Figure 1). This showed that there was no damage to hepatic architecture. Liver sections of rats treated with paracetamol control showed cloudy swelling of fat droplets, very severely degenerated hepatocytes, very severely conjested sinusoids and damaged central vein (Figure 2). This could be due to the formation of highly reactive radicals because of oxidative threat caused by paracetamol. Liver section of rats treated with methanolic extracts of *A. cepa* at 200 mg/kg showed distorted hepatic architecture, severely congested central vein, moderately degenerated hepatocyte and severely congested sinusoid (Figure 3) whereas at 300 mg/kg it showed distorted hepatic architecture, severely congested central vein, moderately degenerated hepatocyte and severely congested sinusoid (Figure 4), furthermore, at 450 mg/kg it showed distorted hepatic architecture, severely congested central vein, moderately degenerated hepatocyte and severely conjested sinusoid (Figure 5). The restorative ability of *A. cepa* on histological architecture could be due to its rich antioxidant effects and its documented effects against reactive oxygen species mediated hepatic damages (Ippoushi et al., 2003; Lee et al., 2009). The liver sections of rats treated with silymarin showed mildly conjested central vein, moderately degenerated hepatocyte and moderately conjested sinusoid (Figure 6). These improved histopathological changes could have been achieved because of the antioxidant effects of silymarin which may have reduced hepatic damage or improved hepatic architecture.

Conclusions

From the results of this experimental study, *A. cepa* showed potent hepatoprotective properties. It was evident that the *A. cepa* was able to reduce significantly all the elevated biochemical parameters due to parace-tamol hepatotoxicity. These encouraging results may have future clinical importance because of the increased use of natural herbs worldwide and Nigeria in particular. Pharmacological evidence at the molecular level is required to establish the actual mechanism of action of the active compounds and its nutraceutical role in human diet.

Conflict of Interests

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

REFERENCES

Achuthan CR, Babu BH, Padikkala J (2003). Antioxidant and hepatoprotective effects of *Rosa damascena*. Pharm. Biol. 41:357-361.

Ahmed RS, Seth VP, Banerjee BD (2000). Influence of dietary ginger (*Zingiber officinale*) on oxidative stress induced by malathion in rats. *Food and Chem.* Toxicol. 38:443-450.

Aniya Y, Miyagi A, Nakandakari N, Kamiya NI, Ichiba T (2002). Free radical scavenging action of the medicinal herb *Limonium wrightii* from the Okinawa islands. Phytomed. 9:239-244.

Babson AI, Babson SR (1973). Kinetic colorimetric measurement of serum lactate dehydrogenase level. Clin. Chem. 19:766-769.

Boyd EM, Hogan SE (1968). The chronic oral toxicity of paracetamol at the range of the LD_{50} (100days) in albino rats. Can. J. Physio. Pharm. 46:239-245.

Chattopadhyay RR (2003). Possible mechanism of hepatoprotective activity of *Azadirachta indica* leaf extract: Part II. J. Ethanopharm. 89: 217-219.

Dama GY, Gore MS, Tare HL, Deore SR, Bidkar JS (2011). Herbal allies for liver protection. *I.* J. Inst. Pharm. Life Sci. 1(1):30-39.

De-Andrade BMA, Soaresa VE, De Souza LM, Sobreira MFR, Cassol DMS, Toma SB (2010). Hepatoprotective treatment attenuates oxidative damages induced by carbon tetrachloride in rats. Exp. Toxicol. Path. 64(3):155-165.

El-Sherbiny EA, Abd-Allah GA, Goneim ST (2003). Relationship of liver function tests to different stages of chronic liver diseases in HCV carriers. J. Egyp. Ger. Soc. Zool: Comp. Physiol. 40:71-93.

Eyo JE, Ozougwu JC, Echi PC (2011). Hypoglycaemic effects of *Allium cepa*, *Allium sativum* and *Zingiber officinale* aqueous extracts on alloxan-induced diabetic *Rattus novergicus*, Med. J. Islam World Acad. of Sci. 19(3):121-126.

Feher J, Cornides A, Cosmos G (1987). Antioxidant and immunomodulant effect of hepatoprotective drugs. In: Okoliesanyi, L., Csomon, G. and Crepaldi, G. (Eds) *Assessment and management of hepatobiliary disease* Heidelberg: Springer-Verlangberlin. pp. 257-263.

Gbile ZO (1980). *Vernacular Names of Nigerian Plants*. Forestry Research Institute of Nigeria, Ibadan.

Gite VN, Deshmukh RD, Sane, RT, Takate SB, Pokharkar RD (2007). Hepatoprotective activity of *Enicostema axillare* against carbon tetrachloride–induced hepatic injury in rats. Pharm. 1:25-30.

Gupta AK, Misra N (2006). Hepatoprotective activity of aqueous ethanolic extract of *Chamomile capitula* in paracetamol hepatotoxic albino rats. Am. J. Pharm. Toxicol. 1(1):17-20.

Hamed MA (2011). Metabolic profile of rats after one hour of intoxication with a single oral dose of ethanol. J. Pharm. Toxicol. 6:158-165.

Hamid A, Budin SB, Mohamed RAP, Manaf NA, Yuhana YN, Husain K, Hamid ZA, Mohamed J (2001). Role of oxidative stress in the protective effects of *Zingiber zerumbet* ethylacetate extract against

paracetamol induced hepatotoxicity in rats. Austr. J. Basic Appl. Sci. 5(8):1519-1525.
Hassan HA, El-Gendy AM (2003). Evaluation of silymarin and / or ginger effect on induced hepatotoxicity by carbon tetrachloride in male albino rats. Egyp. J. Hosp. Med. 12:101-112.
Horvath ME, Gonzalez-Cabello R, Blazovies A, Van Der LM, Barta I, Muzes G, Gregely P, Feher J (2001). Effect of silibinin and Vitamin E on restoration of cellular immune response after partial hepatectomy. J. Ethnopharm. 77(3):227-232.
Ippoushi K, Azuma K, Ito H, Horie H, Higashio H (2003). 6-Gingerol inhibits nitric oxide synthesis in activated mouse macrophages and prevents peroxynitrite induced oxidation and nitration reactions. Life Sci. 73(26):3427-3437.
Iqbal JM, Dewan FZ, Chowdhury SA, Mamun MIR, Moshiuzzaman M, Begum M (2007). Pretreatment by N-hexane extract of *Phyllanthus niruri* can alleviate paracetamol induced damage of the rat liver. Bangl. J. Pharm. 2:43-48.
King PR, King EJ (1984). Estimation of alkaline phosphatase. J. Clin. Pathol. 7:322-325.
Lee TY, Lee KC, Chen SY, Chang HH (2009). 6- Gingerol inhibits ROS and INOS through the suppression of PKC-A and NF–KB pathways in Lipopolysaccharide stimulated mouse macrophages. Biochem. Biophys. Res. Comm. 382(1):134- 139.
Mallory HT, Evelyn KA (1937). The determination of bilirubin with the photometric colorimeter. J. Biol. Chem. 119:481-490.
Mitchell JR, Jollon DJ, Potter WZ, Davis DC, Gillette JR, Brodie BB (1973). Acetaminophen-induced hepatic necrosis, role of drug metabolism. J. Pharm. Exper. Therap. 187:185-194.
Mitra SK, Venkataranganna MV, Sundaram R, Gopumadhavan S (1998). Effect of HD-03, a herbal formulation, on the antioxidant defense system in rats. Phyto. Res. 12:114-117.
Naziroglu M, Cay M, Ustundag B, Aksakal M, Yekeler H (1999). Protective effects of vitamin E on carbon tetrachloride induced liver damage in rats. Cell Biochem. Funct. 17:253-259.
Ozougwu JC, Nwachi UE, Eyo JE (2008). Comparative hypolipidaemic effects of *Allium cepa, Allium sativum* and *Zingiber officinale* aqueous extracts on alloxan-induced diabetic *Rattus novergicus*. Bio –Res. 6(2):384-391.
Ozougwu JC (2011). Anti-diabetic effects of *Allium cepa* (Onions) aqueous extracts on alloxan-induced diabetic *Rattus novergicus*. J. Med. Plants Res. 5(7):1134-1139.
Plaa GL, Hewitt WR (1982). Biotransformation products and cholestasis. Prog. Liver 7:179-194.
Rafi KP, Aleemuddin MA, Sravani K, Krishna KS (2013). Hepatoprotective and antioxidant activity of *Coldenia procumbens* on paracetamol induced liver damage in rats. Eur. J. Zool. Res. 2(4):25-31.
Reddy TS, Shama KP, Nirmala P, Shastry CS (2012). Biochemical studies on hepato and nephroprotective effect of butterfly tree (*Bauhinia purpurea*) against acetaminophen induced toxicity. Intl. J. Res. Ayur. Pharm. 3(3):455-460.
Reitman S, Frankel S (1957). Colorimetric method for the determination of serum transaminases. Am. J. Clin. Path. 28:56-61.
Shaik R, Manisha RL, Suresh BS, Rajesh P (2012). Hepatoprotective activity of alcoholic and aqueous extacts of *Allium cepa linn* (Liliaceae) in rats. Int. J. Pharm. Sci. Res. 3(9):3189-3195.
Rusu MA, Tamas M, Pulca C, Roman I, Sabadas M (2005). The hepatoprotective action of ten herbal extracts in carbon tetrachloride hepatotoxic liver. Phytother. Res. 19:744-749.
Sabina EP, Samuel J, Rajapparanya S, Patel S, Mandal N, Pranatharthuiharan P, Mishra PP, Rasool M (2009). Hepatoprotective and antioxidant potential of *Spirulina fusiformis* on acetaminophen-induced hepatotoxicity in mice. Intl J. Integ. Biol. 6(1):1-5.
Sumy AK, Jahan N, Sultana N, Amin SMR (2011). Effects of Oyster mushroom (*Pleurotus florida*) on paracetamol induced changes of serum bilirubin level and liver tissue protein in rats. J. Bangl. Soc. Physio. 6(1):10-15.
Yuvaraj P, Subramoniam A (2009). Hepatoprotective property of *Thespesia populnea* against carbon tetrachloride induced liver damage in rats. J. Basic Clin. Physio. Pharmacol. 20:169-177.

African Journal of Biotechnology

Related Journals Published by Academic Journals

- *Biotechnology and Molecular Biology Reviews*
- *African Journal of Microbiology Research*
- *African Journal of Biochemistry Research*
- *African Journal of Environmental Science and Technology*
- *African Journal of Food Science*
- *African Journal of Plant Science*
- *Journal of Bioinformatics and Sequence Analysis*
- *International Journal of Biodiversity and Conservation*

academicJournals