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

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

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African Journal of Biotechnology

*Review*

# Microbial agents against *Helicoverpa armigera*: Where are we and where do we need to go?

**Rajendran Vijayabharathi, Bhimineni Ratna Kumari and Subramaniam Gopalakrishnan***

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

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**Plants are prone to various biotic stresses in nature by bacteria, viruses, fungi, parasites, harmful insects and weeds. The biggest percentage loss (70%) in plants is attributed to insects. Lepidoptera is one such diversified phytophagous insect group, which include *Helicoverpa armigera,* a key pest of many food crops including chickpea, pigeonpea, pea, lentil, chillies, sunflower, tomato, tobacco and cotton. Controlling this insect has been a big task for farmers leading to the manufacture of a plethora of pesticides. However, over reliance on chemical pesticides has resulted in problems including safety risks, environmental contamination, outbreaks of secondary pests, insecticide resistance and decrease in biodiversity. Hence, there is an urgent need for the development of eco-friendly methods such as entomopathogens, antagonist or competitor populations of a third organism and botanicals to suppress *H. armigera*. Also, many compounds from microorganisms have been found to be effective in crop production, and these have a role in controlling *H. armigera*. The actinomycetes play an astounding role in controlling the key plant pathogens. They are the representative genera of higher microbial mass in the soil. Numerous studies have shown that these productive actino-bacteria can generate an impressive array of secondary metabolites such as antibiotics, antitumor agents, insecticides etc. This review emphasizes the mechanism behind resistance to insecticides along with actinomycetes and its potential as a biocontrol agent against *H. armigera*.**

**Key words:** *Helicoverpa armigera*, actinomycetes, biocontrol, metabolites.

## INTRODUCTION

Since the commencement of agriculture about 10 000 years ago, insect pests have been a major problem for crop production. The situation still exists and is crucial to manage them due to the rising populations. Malnourishment due to scarcity of food and feed is a major problem especially in poor countries. A recent survey has reported that there are 3.7 billion malnourished people in world (WHO, 2005). Hence, it is important to control crop damages to maintain the quality and abundance of food, feed and fibre around the world. Different approaches namely: chemical based control, host-plant resistance, Integrated Pest Management (IPM) strategies and transgenic plants may be used to prevent, mitigate or control plant infections. The agriculture related pest and patho-

*Corresponding author. E-mail: s.gopalakrishnan@cgiar.org.

gens known so far include 2000 species of insects (Revathi et al., 2011). FAO has reported a worldwide loss of US$120 billion in crop yield, where 20 to 40% was caused by the attack of insect pests and pathogenic organisms (Zhou, 2001). There is a close relationship between insect and host interaction, which leads to crop damage. Much of the plant source (leaves and flowers) has been exploited as food by insects preventing normal growth and products of the plant. The major damaging insect species belong to Lepidoptera (Pimental, 2009). Insect pests affect the food productivity by either generating diseases or by reducing the quality and quantity of food. Low intensification in agriculture on a global level is the root cause for the enhanced damage. An extensive research resulted in broad spectrum chemical insecticides, which have been a primary control agent. About 40% of the insecticides were targeted towards Lepidopteran insects (Srinivasan et al., 2006). An outstretched usage led to pesticide-resistant insects, a reduction in the beneficial insect populations, and harmful effects to humans and the environment (Brooks and Hines, 1999). These problems have encouraged researchers to develop different insect control strategies using both synthetic and natural molecules that are more eco-friendly.

## *HELICOVERPA ARMIGERA* (HUBNER), THE CHALLENGING PEST

*H. armigera* (Kingdom: Animalia, Phylum: Arthropoda, Class: Insecta, Order: Lepidoptera, Family: Noctucidae) is the key pest of agriculture and horticulture in many parts of the world. These pests behave like 'eating machines' and have rapid growth and metabolism (Haq, 2004). The pest status is rooted in its mobility, polyphagy, high reproductive rate, diapause and high fecundity. These factors make it particularly well adapted to exploit transient habitats such as man-made agro-ecosystems. Its predilection for harvestable parts of essential food and high-value crops like cotton, tomato, pulses and tobacco confers a high economic cost to its depredations. It infects dianthus, rosa, pelargonium, chrysanthemum and a range of monocot and dicot crops as well. As per the survey of European Plant Protection Organization (Chamberlin, 2004), *H. armigera* has been widespread in Asia, Africa and Oceania. In India, *H. armigera* commonly destroys over half the yield of pulse crops like pigeon pea and chickpea, which leads to $US 300 million loss per annum (EPPO, 2006), while in the late 1980s losses of both pulses and cotton were estimated to exceed US$500 million in addition to the investment of US$127 million in insecticides.

The mid-gut epithelium is large in *H. armigera* and effectively digests and absorbs nutrients, which is responsible for its rapid growth (Reed and Pawar, 1982). *Helicoverpa* females lay eggs singly on leaves, flowers and young pods. The larval form of the insect feeds on the foliage (young leaves) in chickpea and a few other legumes, whereas it feeds on flowers and flower buds in the case of cotton and pigeonpea. Young seedlings of chickpea are destroyed completely when this larva feeds on them. Plants in tropical climates such as the southern part of India are more prone to these insects. Larger larvae bore into pods/bolls and consume the developing seeds inside the pod. With such dire scenario in India, farmers try to control this pest by various classes of insecticides.

## MANAGEMENT TACTICS OF *HELICOVERPA*

Synthetic pesticides have been in use for the last 50 years, irrespective of pest types in the field and under post-harvest conditions. For high productivity of crops it was found crucial to apply massive amounts of pesticides to control the pest (Chamberlin, 2006), but this leads to disturbance in the ecology as a huge number of pest and predators have been killed by these pesticides, thereby giving way to secondary pests (Armes et al., 1996). The outbreak of plant damage in 1980 was one such example, which was controlled by overuse of pesticides. This led to the resistance of *H. armigera* against many conventional insecticides such as organochlorine, organophosphate, carbamates and pyrethroid insecticides. These insecticides are broad spectrum neurotoxic with a wide host range from insects to mammals. Many researchers have found that this toxicity acts over insects and also non-target groups of organisms (Jiang and Ma, 2000; Rai et al., 2009). The insect pest on cotton crop in India became tolerant to organophosphate insecticides, which triggered the process of re-thinking about the use of chemicals for pest control. After 1980s, a new concept of pest management evolved, called IPM, which combines the methods of cultural, mechanical, biopesticides and chemical pesticides. A case study by Krishi Vignan Kendra, Medak, India, highlighted some non-pesticidal methods followed by farmers such as deep ploughing, burning of farm refuge, intercropping or mixed cropping, trap cropping, bird perches (to attract predators), manual collection and destruction of larvae, natural extracts, mechanical collection, and collection with pheromone traps.

## MECHANISM OF INSECTICIDE RESISTANCE

The mechanism of insecticide resistance has been reported on various factors. All the mechanisms have elucidated that the foremost factors for resistance are decreased insensitivity, reduced penetration and metabolic detoxification (El-Latif and Subrahmanyam, 2010). The resistance of *H. armigera* against pesticides has been extensively studied. The strains that are resistant were

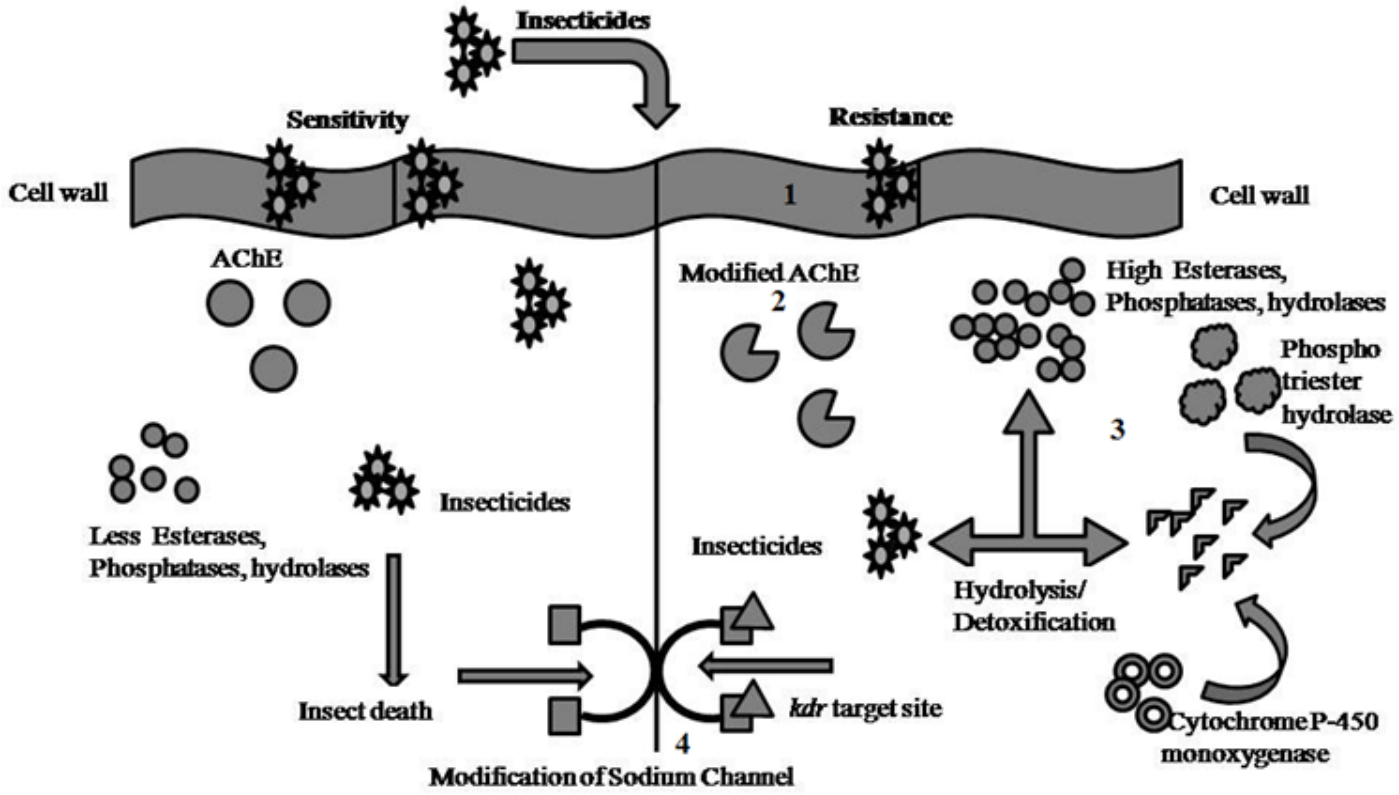


**Figure 1.** Schematic diagram of all possible mechanism of insecticide resistance in comparism with sensitive insects. **1.** Reduced penetration. **2.** Acetyl choline esterase (ACHE) modification. **3.** Metabolic detoxification or sequestering of insecticide by group of enzemes (esterase, phosphoteriester hydrolase, cytochrome P-450 monooxygenase) **4.** Modification of sodium channel conferring kdr target site resistance to pyrethroids.

found to have higher production of esterases (Scott, 1991) and glutathione *S*-transferases. The most common type of resistance involves the target site insensitivity (*kdr*) mechanism and increased enzymatic detoxification. The cytochrome P-450-dependent monooxygenase is an extremely important and most frequent type of metabolic system that also influences resistance (Ellison et al., 2011). The possible mechanism of insecticide resistance in *H. armigera* is depicted in Figure 1. The problem allied with synthetic insecticides has led to the search for a sustainable alternative in pest control. This can be substantiated by the growth of organic farming and banned notices of synthetic pesticides in many areas (Christie, 2010). One among the alternatives for pest control is the biopesticides, which had a global market value of approximately US$1 billion in 2010, and which will increase to US$3.3 billion in 2014 (Lehr, 2010).

## BIOLOGICAL PESTICIDES FROM TREASURE BOX

For decades natural products have been used for humankind either for food, clothing, cosmetics, shelters, traps, tools, weapons and antibiotics or crop protection agents. Initially, crude mixtures of chemicals were isolated from microbes and used to some extent, but recently, advanced scientific methods have led to the development of pure products, which act effectively (Copping and Duke, 2001). Scientists all over the world are averse to synthetic pesticides and have now diverted their attention to the development of safe and more permanent methods of pest control. They have found that biological control is one of the best options as it is eco-friendly and can be integrated with other pest management strategies. Hence, biological pesticides have been used to manage *H. armigera* (King and Coleman, 1989). Use of parasitoids and predators has failed to be successful pest control methods because of constraints in their mass production, storage and availability to the farmers on time. These circumstances have moved the focus to microorganisms.

Microbes are treasure boxes that contain pharmacoactive drugs and antibiotics. A new arena with microbial pesticides isolated from beneficial microbes has been introduced for crop protection. Viruses, bacteria and fungi can act as biocontrol agents against insects and fungi. The mode of infection of virus and bacteria is via their digestive tract while fungi make entry into the host through

the cuticle (Deshpande, 1999). Investigation of our research group on PGP bacteria *B. subtilis*, *B. megaterium, Serratia mercescens* and *Pseudomonas* spp.; fungus *Metarhizium anisopliae* and actinomycetes *S. cavourensis* sup sp. *cavourensis, S. albolongus, S. hydrogenans, S. antibioticus, S. cyaneofuscatus, S. carpaticus, S. bacillaris* and *Streptomyces* spp. isolated from herbal vermicomposts and organically cultivated fields documented the broad-spectrum insecticidal of all these microbes against lepidopteran pests such as *H. armigera, Spodoptera litura* and *Chilo partellus* (Gopalakrishnan et al., 2011; Vijayabharathi et al., 2014). This proves the efficiency of microbes as treasure box for biological pesticides.

## FUNGAL INSECTICIDES

The study of microbes as insecticides has brought to light that fungal populations are efficient producers of ubiquitous defensive proteins called lectins. Though plants themselves produce these proteins, they have a limitation on nonspecific activity, hence fungal lectins were concentrated upon (Carlini and Grossi-de-Sa, 2002; Peumans et al., 2000). At a suitable concentration, fungi like *Beauveria bassiana*, *Lecanicillium lecanii M. anisopliae, Nomuraea rileyi, Paecilomyces* spp. were proven to show an effective reduction in the number of insect pests (Ambethgar, 2009). However, the success of these fungi depends on the conidial viability (Olivera and Neves, 2004) because germination decides the pathogenesis (Alizadeh et al., 2007).

The efficacy of fungal pathogen *N. rileyi* (Farlow) Samson has been proven against *H. armigera* by a number of researches in groundnut, soybean and cotton ecosystems (Patel, 2001; Hegde et al., 2004; Ramigowda, 2005). Since *N. rileyi* is a facultative fungus, it can be easily multiplied on rice or sorghum grains under laboratory conditions, and can be utilized in the management of this polyphagous pest. Shekarappa (2009) conducted a field study with *N. rileyi* in sorghum against *H. armigera* and found that mycoinsecticide *N. rileyi* was highly infective at both dosages of $1 \times 10^8$ conidia per litre and $2 \times 10^8$ conidia per litre against *H. armigera* compared to the chemical control malathion in mitigating the pest population as well as in obtaining higher yields. The fungi *N. rileyi* and *Isaria tenuipes*, pathogens for lepidopteran were evaluated for their activity of oil suspended conidia against *Helicoverpa zea,* and this could also be successful against *H. armigera* (Aquinoa et al., 2010).

*M. anisopliae* and *B. bassiana* were found to be less efficacious insecticides on chickpea (Kale and Men, 2008), but they were more potent on combinations with nucleopolyhedrosis virus and *Bacillus thuringiensis (Bt)*. Ensuring survival and persistence of the fungi is one of the greatest challenges in using fungal insecticides, where optimization strategies are lacking in this arena. It has been recorded that about 500 (Davis, 1996) host species of Lepidoptera and 200 (Zimmermann, 1993) other insect families have been infected by two types of fungi, white muscardine (*B. bassiana)* and green muscardine (*M. anisopliae)*, respectively. The chitin deacetylase of *M. anisopliae* was found to contribute to pathogenesis. This initiates the infection by softening the insect cuticle to aid penetration (Nahar et al., 2004). Enzyme-based biocontrol agent from the fungus *Trichoderma harzianum* was also evaluated against the growth and metamorphosis of *H.armigera* (Pandey et al., 1999).

## VIRAL INSECTICIDES

The narrow host range of most viruses can be both a limitation and an advantage depending on the pests. Viral insecticides are effective biological controls used with insect-specific nucleopolyhedrosis viruses (NPVs). These NPVs are highly virulent and lethal, but are slow acting. Baculoviruses consist of two genera, nuclear polyhedrosis virus (NPVs) and granulo virus (GVs), and are host-specific (Black et al., 1997; Van Reomurnd et al., 1997). The virus specific to *H. armigera,* a single nucleocapsid nucleopolyhedrosis virus (HaSNPV), is used commercially, which is a promising alternative since they are target-specific and highly pathogenic (Moscardi, 1999).

Recent trials in Queensland carried out by Commonwealth Scientific and Industrial Research Organization (CSIRO) Entomology, University of Queensland and the Queensland Department of Primary Industry on sorghum have suggested that NPV produced *in vitro* will also be effective as much as NPV cultured through *in vivo*. Although NPV is a promising insecticide, some constraints have made them difficult to use. The slow activity of the pesticides is improved by genetic modifications to generate fast killing (Inceoglu et al., 2001). Recombinant HaSNPV has been recently introduced with gene deletion or insertion, which could act at a high speed. Bioassays of these recombinants have shown that they could kill the second instar *H. armigera* at a faster rate than the wildtype (Chen et al., 2000; Sun et al., 2004a). However, these genetically modified viruses are ecologically not successful due to its limited production of polyhedral and alteration in the behaviour of larvae (Sun et al., 2004b; Zhou et al., 2005).

Entomopox virus (EPV) is also found to be a potent insecticide. They have a very small range of hosts and hence they do not affect non-target organisms. Current research is ongoing on EPV's special protein component, which severely restricts the growth of pest caterpillar, reduces their appetite and thus may result in less damage to the crop. Time taken for effective killing is the limitation of this EPV, and hence an improved technology for speeding the process is needed. Stunt viruses (SVs) are also emerging as insecticides that have a small compact RNA and can therefore self-assemble from their components. This helps in producing them *in vitro.* Expe-

riment trials should be further carried out for their efficacy (Mettenmeyer, 2002). Scientists are more confident about these virus insecticides, which prove to have no adverse effects on the environment. Yet, speeding up the control stage and improving success rates at the field level are very important and need to be.

## BACTERIAL INSECTICIDES

Bacillus genera in the bacterial family are found to be effective insecticides from a decade ago. The proteins present in *B. thuringiensis* are found to possess insecticidal properties and show efficacy as spray formulations in agriculture. They have been expressed in crop plants. *B. thuringiensis* (Bt) has been developed to be a potent biopesticide. *Bt* is Gram-positive and sporulating soil bacterium. The proteins such as d-endotoxins and cytolytic proteins produced by *Bt* are efficient sources of insecticides for food crops and stored grains (Meadows, 1993). The Cry toxin of Bt is one such endotoxin that acts against a wide range of insects (Federici, 2005). The gut regions of these pests are targeted by the toxins causing death by starvation (Starnes et al., 1993). Many transgenic plants have been developed that can produce insecticidal proteins that are derived from this genera. They have been successfully used in control of pest and protecting important high acre crops (Perlak et al., 2001; Pilcher et al., 2002). Cry toxins are of various types (a, b, c etc.) and differ in their host specificities. *Cry 1 Ab* and *Cry 1 Ac* genes were used in the first commercial genetically modified (GM) cotton and corn crops that were produced against Lepidopteran pests (Llewellyn et al., 2007).

There is a long lasting argument that transgenic plants can affect the natural environment. It is obvious that a negative impact can also be created by interference from natural protection. However, severe problems have also occurred in Bt-transgenic crops where pest insects had gained resistance to Bt-toxin just as in the development of the resistance to many chemical insecticides. The most common mechanism of resistance is by the disruption of binding of Bt toxin to receptors in the mid-gut membrane. This disruption might be either due to mutations in the receptors or changes in the expression of the receptors (Fuentes et al., 2011; Tiewsiri and Wang, 2011). The resistance mechanism associated with ABC transporter loci has also been reported (Baxter et al., 2011).

Approaches like ‘high dose/refuge strategy’ (Chilcutt and Tabashnik, 2004) or pyramid by expression of two genes have been tried to prolong the effectiveness of *Bt*-crops (Suresh et al., 2008). Another severe issue is on the transfer of inserted transgenes from crops to wild or weedy plants. Many control measures on transgene flow have been performed with special reference to plastid transformation, but still there is a problem of relocation of plastid into the nuclear genome itself (Huang et al., 2003). *Bt* plants also have little effect on soil biota and hence further research is needed to know the effects of GM plants on soil decomposition. Assessment of non-target impacts is an essential part of risk assessment in insect-resistance GM plants (Ócallaghan et al., 2005). There are still miles to travel for the success of transgenic plants, with solutions that dispel all negative impacts.

## ACTINOMYCETES AND ITS METABOLITES

Actinomycetes come under the order actinomycetales, which are Gram positive bacteria with high G+C content. They are wonderful resources of biologically active secondary metabolites that are important for chemical, pharmaceutical and agricultural industries. Actinomycetes are omnipresent in nature, but prefer soil components (Lechevalier et al., 1981). They therefore play an important role in soil biodegradation and humus formation (McCarthy and Williams, 1992; Stach and Bull, 2005). These are the bacteria responsible for the “wet earth odour” that emanates from wet soil due to volatile substances like geosmin (Wilkins, 1996) and are potent producers of extracellular enzymes (Sehrempf, 2001). To date, about 20 different genera of actinomycetes have been isolated and many are to be explored (Williams and Wellington, 1992). Actinomycetes produce a variety of antibiotics with diverse chemical structures such as polyketides, β-lactams and peptides in addition to a variety of other secondary metabolites that have antifungal, antitumor and immune suppressive activities (Behal, 2000).

Microbial cells produce synthetases that catalyse transformation of unused substances into molecules called secondary metabolites. Secondary metabolites consist of natural products that (a) are restricted in taxonomic distribution, (b) are synthesized for a finite period by cells that have stopped dividing, and (c) most probably function as convenient disposal packages of excess primary substances. Some of these metabolites act as toxins for non-producer cells. Included in this category are hormones, pheromones, toxins and antibiotics. Substrates of secondary metabolism are primary metabolites as acetate, pyruvate, malonate, mevalonate, shikimate, prephenate, amino acids and purines (Demain, 1992; 1995).

The genus *Streptomyces* is the prime group that comes under actinomycetes. About 70% of the explored actinomycetes were found to be species of *Streptomyces*. They have the capacity to produce significant compounds, especially antibiotics, insecticides and pigments, due to their extra-large DNA complement (Goodfellow and Williams, 1986). They follow a special metabolic pathway, which includes the formation of glycosides and uses the shikimate pathway to aromatic compounds. The distribution of natural products in *Streptomyces* is attributed by the antibiotic biosynthetic gene transfer (Baltz, 2005). Overall 23,000 bioactive components have been reported, of which 10,000 compounds are produced by actino-

mycetes. Of this, 7600 compounds are reported to be from *Streptomyces* species (Berdy, 2005).

The next important genus of actinomycetes is *Micromonospora*. They are best known for synthesizing antibiotics, especially aminoglycoside, enediyne and oligosaccharide antibiotics, and hence they are employed in biocontrol. These genera are yet to be fully explored, after which new light on actinomycetes population might emerge. The secondary metabolites of actinomycetes, namely tetranectin, avermectins, faerifungin and macrotetrolides and flavonoids produced were found to be toxic to many insects. Avermectins are compounds produced by a novel species *S. avermitilis* isolated from soil. They were found to be an effective antihelminthic compound earlier (Burg et al., 1979), but later it was also found to be a potent insecticide, acaricide and nematicide (Putter et al., 1981).

Spinosyn is a large family of unprecedented compounds isolated from two species of *Saccharopolyspora spinosa*. The fermentation of *S. spinosa* produces several metabolites that are called spinosyn A and spinosyn D. They have a novel molecular structure and their mode of action is by affecting nicotinic acetylcholine receptors at the post-synaptic cells. They are very selective towards target insects such as Lepidoptera and Diptera, but generally show very low activity against many beneficial insect predators and non-target species (Thompson, 2000; Salgado and Sparks, 2005). The efficiency of spinosa depends on the type of species and their stage of development, exposure time and method of administration. The significant advantage of spinosad also includes less toxicity towards mammals, avians and aquatic organisms compared to other insecticides, thus making it safer to use (Thompson et al., 2002). Several studies have shown that spinosyn has no long time persistence ability in plants, soil and other environments due to facile degradation by microbes present, whereby they partition them into organic matter and sediment with subsequent biotic degradation in the absence of light. Overall, they are easily subjected to diverse degradative pathways and metabolic mechanisms, and hence reduce their persistence in plants, and other environments (Kirst, 2010).

## INSECTICIDAL AND LARVICIDAL PROPERTIES OF ACTINOMYCETES

Many studies have used insecticidal and larvicidal compounds extracted from actinomycetes. Larvicidal actinomycetes were explored from marine samples of Muthupet Mangrove, Tamilnadu (Vijayakumar et al., 2010). The actinobacterial extracts isolated from the marine source showed larvicidal effects on 24 h exposure at 1000 ppm. The highest larval mortality was detected in LK-3 extract against the larvae of *Culex gelidus* with an LC50 of 108.08 ppm and against *Culex tritaeniorhynchus* at LC50 of 146.24 ppm (Karthik et al., 2011). Valinomycin has been reported to be an insecticidal antibiotic by *Streptomyces griseus* var. *flexipertum* var. nov (Heisey et al., 1988). *Streptomyces nanchangensis*, a producer of nanchangmycin, was found to be an insecticidal polyether antibiotic. *Streptomyces lavendulae* is another promising species known to release many secondary metabolic compounds that are considered as important sources of antibiotics and pesticides. The antitumor antibiotic mitomycin C (MC) produced by *S. lavendulae* is a bioreductively activated alkylating agent that crosslinks DNA at 5'CpG sequences. It has been widely used clinically for antitumor therapy (Johnson et al., 1997). A high molecular weight transglutaminase inhibitor has also been purified from the culture filtrate of *S. lavendulae* Y-200 (Ikura et al., 2000). *S. lavendulae* SNAK 64297 releases a novel compound, 1100-50 (1), which has been isolated and purified by Takatsu (Takatsu et al., 2003). All these antibiotics are found to have insecticidal properties. A list of compounds recently found to be with potent pesticidal or larvicidal properties has been shown in Table 1 along with the source of the compound.

## CONCLUSION

There is an increasing demand to discover environment friendly insecticides and pesticides in general. *H. armigera* had made this situation still shoddier because of its voracious feeding habit and the extent of damage it can cause to the agricultural community. Therefore, research has been forced in this area where an effective alternative is needed. This review provides handy knowledge of our treasure box microbes with special reference to actinomycetes. With a push for cleaner and greener alternatives to traditional chemicals, insecticides from microbial sources, particularly actinomycetes, will be strong competitors in the future insecticide market. There is a wide diversity of actinomycetes that are still unexplored and hence a detailed study on these diverse valuable actinomycetes by culturable and unculturable methods will lead to the discovery of novel insecticidal and larvicidal compounds. Few studies have started flourishing in isolation and characterization of the compounds from actinomycetes. Advanced techniques like matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) are employed to characterize these compounds from actinomycetes (Stafsnes et al., 2012). To exploit these findings for agricultural use, a typical field study and strategies of optimization are necessary.

Many strategies have been applied for improvement of secondary metabolites, which include pathway-specific, or pleiotrophic regulators, or enhancing the availability of precursors, ribosomal engineering etc. Recent interest has been focussed on altering the secondary metabolism through addition of small molecules, which has significant advantages. Bacteriophages are also found to be used

**Table 1.** Microbial compounds with insecticidal and larvicidal properties

| Source | Compound | Activity | Reference |
|---|---|---|---|
| *Streptomyces nanchangensis NS3226* | Nanchangmycin | Insecticidal | Sun et al., 2002 |
| *Streptomyces* spp. CP1130 | Tartrolone C | Insecticidal | Lewer et al., 2003 |
| *Streptomyces galbus* | Ethyl acetate extract | Pesticidal | Jo et al., 2003 |
| *Streptomyces* spp.173 | Fermented broth | Insecticidal | Xiong et al., 2004 |
| *Metarrhizium* spp. FKI-1079 | Hydroxyfungerins A & B | Insecticidal | Uchida et al., 2005 |
| *Streptomyces qinlingnensis*.nov. | Fermented broth | Insecticidal | Zhi-qin et al., 2007 |
| *Streptomyces* spp.4138 | Staurosporine | Insecticidal | Xiao-ming et al., 2008 |
| *Streptomyces* spp.KN-0647 | Quinomycin A | Insecticidal | Liu et al., 2008 |
| *Streptomyces* spp. ERI-04 | Curde extract | Antifeedant | Valanarasu et al., 2010 |
| *Streptomyces microflavus* | Crude extract | Larvicidal | El-Bendary et al., 2010 |
| *Saccharomonospora* spp. (LK-1), *Streptomyces roseiscleroticus* (LK-2), & *Streptomyces gedanensis* (LK-3) | Crude extract | Larvicidal | Karthik et al., 2011 |
| *Streptomyces* spp. CMU-MH021 | Fervenulin | Nematocidal | Ruanpanun et al., 2011 |
| *Streptomyces microflavus* neau3 | Macrocyclic lactone | Insecticidal | Wang et al., 2011a |
| *Serratia marcescens* NMCC46 | Prodiogisin | Larvicidal | Patil et al., 2011 |
| *Streptomyces avermitilis* NEAU1069 | Doramectin congeners,1-4 | Acaricidal & insecticidal | Wang et al., 2011b |
| *Streptomyces* spp. | 2-Hydroxy-3,5,6-trimethyloctan-4-one | Larvicidal | Deepika et al., 2011 |
| *Chromobacterium violaceum* ESBV 4400 | Violacein | Larvicidal & pupicidal | Baskar and Ignacimuthu, 2012 |
| *Streptomyces* spp,VITSVK5 | 5-(2,4-Dimethylbenzyl) pyrrolidin-2-one (DMBPO) | Larvicidal | Saurav et al., 2011 |
| *Saccharopolyspora pogona* | Butenylspinosyn | Insecticides | Lewer et al., 2009 |

*Corresponding author. E-mail: s.gopalakrishnan@cgiar.org. Tel: +91 40 30713610.

as a powerful tool in the detection of bioactive actinomycetes and help in discovering the novel bioactive compounds. This offers a more significant benefit if improved understanding host-phage ecology is known; hence a sound knowledge on microbial taxonomy is necessary for the effective use of bacteriophage as a tool in the selective isolation procedure. Therefore, we could go far long to meet the major challenges in pest management with biotechnological tools. Microbes will continue to offer valuable versatile products, thereby serving mankind. The authors hope that this review will fill the gap with knowledge still lacking in this area.

## Conflict of Interests

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

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*Full Length Research Paper*

# Cloning of partial *cry1Ac* gene from an indigenous isolate of *Bacillus thuringiensis*

**R. Manikandan, A. Ramalakshmi, V. Balasubramani and V. Udayasuriyan***

Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore-641 003, Tamil Nadu, India.

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**The discoveries of novel *cry* genes of *Bacillus thuringiensis* (Bt) with higher toxicity are important for the development of new products. The *cry1* family genes are more toxic to the lepidopteran insects according to the previous reports. In the present study, nine indigenous isolates of Bt were used for screening of *cry1* genes by PCR using degenerate primers specific to *cry1* family genes. Two of the nine new isolates of Bt showed amplification with *cry1* family primers. Partial *cry1*gene(s) was cloned from one of the *cry1* positive isolate of Bt, T133. Nucleotide sequence data generated for partial *cry1* gene of T133 showed 98% homology with 1420 bp of the partial *cry1Ac1* gene. Deduced amino acid sequence of the partial *cry1Ac* of Bt strain, T133 showed two per cent variation in comparison to Cry1Ac1 by differing at 5 positions; one deletion at 442th position and five substitutions at the following positions, 296, 367, 440 and 563.**

**Key words:** *Bacillus thuringiensis, cry1* genes, degenerate primer, cloning.

## INTRODUCTION

The control of agriculture pest populations is achieved mainly by the application of chemical insecticides; the continuous use of synthetic pesticides led to serious problems like environmental degradation and development of resistance in insect pest (Shelton et al., 2002). Recently, there has been a renewed interest in the development of biological alternatives to chemical pesticides. The *Bacillus thuringiensis* (Bt) Berliner is considered as one of the most versatile microbial insecticides. It is a gram-positive spore-forming soil bacterium. The insecticidal activity is based on the ability of the bacterium to produce large quantity of larvicidal proteins known as delta-endotoxins (Cry proteins). The Cry toxins constitute a family of related proteins that can kill insects belonging to the Lepidoptera, Coleoptera, Diptera, Hymenoptera, Homoptera and Mallophaga, as well as other invertebrates (Schnepf et al., 1998; Feitelson et al., 1999).

*Corresponding author. E-mail: udayvar@yahoo.com. Tel: 91-422-6611353.



**Abbreviations: Bt,** *Bacillus thuringiensis*; **PCR,** polymerase chain reaction; **IPM,** integrated pest management; **IPTG,** isopropyl thiogalaactoside; **LB,** Luria-Bertani; **cry,** crystal protein.

**Table 1.** Primers used for screening and amplification of *cry1* gene fragments.

| Primer name | Sequence (5' to 3') | Amplicon size (bp) |
|---|---|---|
| JF | MDATYTCTAKRTCTTGACTA | ~1500 to 1600 |
| JR | TRACRHTDDBDGTATTAGAT | |

B = C, G or T; D = A, G or T; H = A, C or T; K = G or T; M = A or C; R = A or G and Y = T or C. Source: (Juarez-Perez et al., 1997).

The advancement in molecular biology led to the cloning of Bt crystal protein (*cry*) gene for the first time in 1981 (Schnepf and Whiteley, 1981). So far more than 445 *cry* genes have been successfully cloned and characterized for their insecticidal potential.

Cry1A toxins are very important because of their high toxicity to lepidopteran pests and widespread distribution among Bt strains (Li et al., 1995; Bravo et al., 1998; Uribe et al., 2003). Variation in toxicity and specificity exist among different Cry1A toxins due to minor amino acid substitutions (Tounsi et al., 1999). About 69 *cry1A* genes are classified into *cry1Aa* to *cry1Ai* sub-types (http:// www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). It is still essential to search for novel Bt strains that may lead to the discovery of additional insecticidal proteins with higher toxicity against wider pest range. The PCR method is proven to be a powerful tool which allows quick, simultaneous screening of many Bt samples, identification of specific insecticidal genes carried by different Bt strains, classification of *cry* genes and subsequent prediction of their insecticidal activities (Carozzi et al., 1991; Ben-Dov et al., 1997; Juarez et al., 1997). The amplification, subsequent cloning and sequence analysis of the *cry* genes from new isolates of Bt are important because they may provide new gene sequences encoding more active toxins which could be used for developing better versions of transgenic crop plants. So, the present study was undertaken for cloning and sequencing of *cry* gene from indigenous isolates of Bt.

## MATERIALS AND METHODS

### Bacterial strains and plasmids

Nine indigenous Bt strains (T17, T21, T50, T75, T81, T133, T134, T142 and T147) and a reference strain, Bt (indigenous strain, 14r1) are from the collection of Bt strains maintained by the corresponding author in the Department of Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, India. *Escherichia coli* vector pTZ57R\T obtained from Fermentas INC.

### Amplification of Bt DNA by PCR

Total genomic DNA from *B. thuringiensis* strains, T17, T21, T50, T75, T81, T133, T134, T142 and T147 were extracted as described earlier by Kalman et al. (1993) and used as the template for the PCR screening. The genomic DNA of a reference strain of Bt, 14r1 was used as positive control. A set of degenerate primers (JF and JR) described by Juarez-Perez et al. (1997) were used to screen the new isolates of Bt for the presence of *cry1* family genes (Table 1). These primers are expected to amplify fragments of ~ 1.5 kb from the *cry1* family genes.

The PCR was accomplished using an Eppendorf thermal cycler in 25 µl reaction volume containing total genomic DNA of Bt (30 ng), 2.5 µl of 10X PCR buffer (10 mM Tris-HCl; pH: 9.0, 50 mM KCl, 1.5 mM $MgCl_2$) 75 µM each of dNTPs, 50 ng each of forward and reverse primers and 0.5 Units of *Taq* DNA polymerase. The PCR was performed for 30 cycles as follows: 94°C for 1 min, 40°C for 45 s, 72°C for 2 min and the final extension was performed for 7 min at 72°C.

### Cloning of partial *cry1* fragments from Bt isolate, T133

The gel eluted PCR products of partial *cry1* gene (~1.5 kb) from Bt isolate, T133 was ligated into T/A vector (pTZ57R\T Fermentas INC) as per the manufacturer's instruction. The ligated mixture was transformed into *E. coli* as per the standard procedure (Sambrook et al., 1989). The transformed colonies of *E. coli* were screened by colony PCR with M13F and M13R primers for checking the presence of insert (partial *cry1* fragment of Bt isolate, T133).

### Nucleotide sequencing of recombinant plasmids

The plasmid DNA was isolated from the *E. coli* transformants containing partial *cry1* gene of Bt isolate, T133 and nucleotide sequence of recombinant plasmids was carried out by automated sequencing (Ist Base, Singapore). The sequence data was subjected to homology search through Basic Local Alignment Search Tool (BLAST) of National Centre for Biotechnological Information (NCBI) (www.ncbi.nlm.nih.gov/Blast). The deduced amino acid sequence was generated by BioEdit (Hall, 1999).

## RESULTS

### Screening of new isolates of Bt for *cry1* genes

Total genomic DNA isolated from indigenous isolates of Bt was used as template for screening of *cry1* genes by PCR using the degenerate primers specific to *cry1* family genes. Amplification of expected size (~ 1.5 kb) of PCR products were obtained from two new Bt isolates T17 and T133, whereas amplification was not observed in the remaining seven isolates (Figure 1).

### Cloning and sequence analysis of partial *cry1* gene from Bt isolate, T133

The gel eluted PCR product (~1.5 kb) of partial *cry1* gene

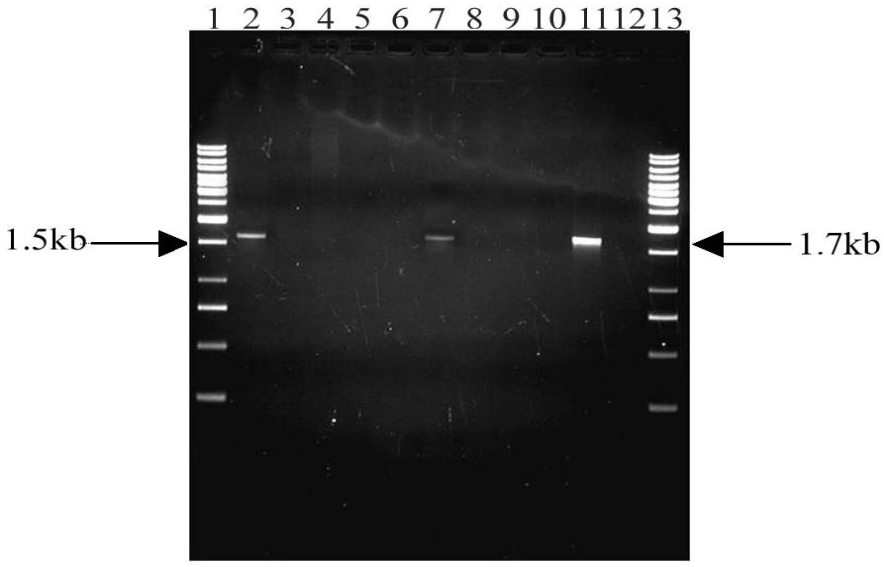


**Figure 1.** Screening of Bt strains for the presence of *cry1* gene by PCR. **Lanes 1 and 13:** 1 kb marker; **lanes 2 and 7:** new isolates of Bt positive for *cry1* gene (T17 and T133); **lanes 3, 4, 5, 6, 8, 9 and 10:** new isolates of Bt negative for *cry1* gene (T21, T75, T81, T134, T142, T147 and T50); **lane 11:** positive control (14r1 genomic DNA) and **lane 12:** negative control.

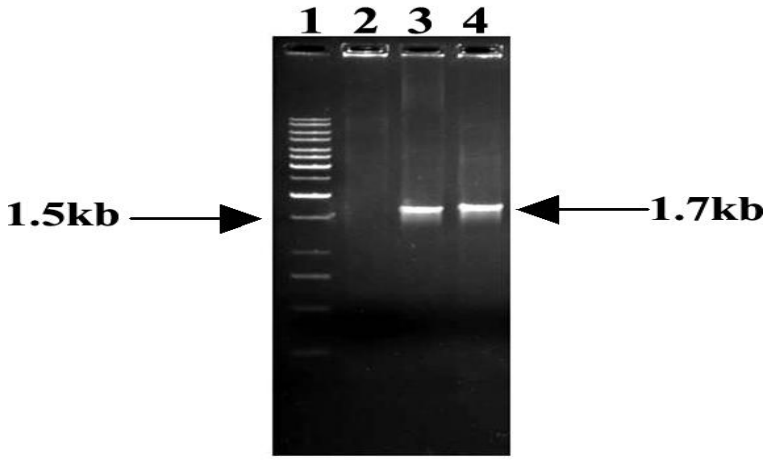


**Figure 2.** Screening of recombinant *E. coli* colonies by PCR for presence of partial *cry1* gene. **Lane 1:** 1 kb marker; **lane 2:** negative control; **lane 3:** partial *cry1* gene fragment from 14r1 (positive control) and **lane 4:** partial *cry1* gene from new isolates of Bt, T133.

fragment from Bt isolate, T133 was cloned into pTZ57R/T vector (T/A vector). The recombinant clones (white colonies) were selected on LB agar containing X-gal, IPTG and ampicillin. Presence of insert was confirmed in recombinant *E. coli* colonies, by colony PCR with M13 forward and M13 reverse primers. Agarose gel electrophoresis of DNA amplified from the transformants of *E. coli* showed expected size of ~1.7 kb corresponding to the sum of insert DNA of 1.5 kb and vector sequence of about 200 bp (Figure 2). Recombinant plasmid isolated from three of the *E. coli* clones were used to determine nucleotide sequence of the partial *cry1* gene of Bt strain,

```
Query  1    RSPHSMDILNSITIYTDAHRGYYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIV  60
            RSPH MDILNSITIYTDAHRGYYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIV
Sbjct  292  RSPHLMDILNSITIYTDAHRGYYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIV  351

Query  61   AQLGQGVYRTLSSTFYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLD  120
            AQLGQGVYRTLSST YRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLD
Sbjct  352  AQLGQGVYRTLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLD  411

Query  121  EIPPQNNNVPPRQGFSHRLSHVSMFRSG-SSSSVSIIRAPMFSWIHRSAEFNNIIASDSI  179
            EIPPQNNNVPPRQGFSHRLSHVSMFRSG S+SSVSIIRAPMFSWIHRSAEFNNIIASDSI
Sbjct  412  EIPPQNNNVPPRQGFSHRLSHVSMFRSGFSNSSVSIIRAPMFSWIHRSAEFNNIIASDSI  471

Query  180  TQIPAVKGNFLFNGSVISGPGFTGGDLVRLNSSGNNIQNRGYIEVPIHFPSTSTRYRARV  239
            TQIPAVKGNFLFNGSVISGPGFTGGDLVRLNSSGNNIQNRGYIEVPIHFPSTSTRYR RV
Sbjct  472  TQIPAVKGNFLFNGSVISGPGFTGGDLVRLNSSGNNIQNRGYIEVPIHFPSTSTRYRVRV  531

Query  240  RYASVTPIHLNVNWGNSSIFSNTVPATATSLNNLQSSDFGYFESANAFTSSLGNIVGVRN  299
            RYASVTPIHLNVNWGNSSIFSNTVPATATSL+NLQSSDFGYFESANAFTSSLGNIVGVRN
Sbjct  532  RYASVTPIHLNVNWGNSSIFSNTVPATATSLDNLQSSDFGYFESANAFTSSLGNIVGVRN  591

Query  300  FSGTAGVIIDRFEFIPVTATLEAEYNLERAQKAVNALFTSTNQLGLKTNVTDYHIDQVSN  359
            FSGTAGVIIDRFEFIPVTATLEAEYNLERAQKAVNALFTSTNQLGLKTNVTDYHIDQVSN
Sbjct  592  FSGTAGVIIDRFEFIPVTATLEAEYNLERAQKAVNALFTSTNQLGLKTNVTDYHIDQVSN  651

Query  360  LVTYLSDEFCLDEKRELSEKVKHAKRLSDERNLLQDSNFKDINRQPERGWGGSTGITIQG  419
            LVTYLSDEFCLDEKRELSEKVKHAKRLSDERNLLQDSNFKDINRQPERGWGGSTGITIQG
Sbjct  652  LVTYLSDEFCLDEKRELSEKVKHAKRLSDERNLLQDSNFKDINRQPERGWGGSTGITIQG  711

Query  420  GDDVFKENYVTLSGTFDECYPTYLYQKIDESKLKAFTRYQLRGYIEDSQDLEI  472
            GDDVFKENYVTLSGTFDECYPTYLYQKIDESKLKAFTRYQLRGYIEDSQDLEI
Sbjct  712  GDDVFKENYVTLSGTFDECYPTYLYQKIDESKLKAFTRYQLRGYIEDSQDLEI  764
```

**Figure 3.** Homology between the deduced amino acid sequence of partial *cry1Ac* gene of T133 and holotype, Cry1Ac1. **Query:** *cry1Ac* amino acid sequence of Bt strain, T133; **Subject:** *cry1Ac1* amino acid sequence. Boldface letters indicate the six amino acid differences between Cry1Ac of Bt strain, T133 and Cry1Ac1 (holotype).

T133 by automated DNA sequencing. Nucleotide sequence data (Acc. No. FJ794964) revealed 98% homology with 1420 bp bases in the 5' region of holotype, *cry1Ac1*. Deduced amino acid sequence of partial Cry1Ac gene showed one deletion at 442th position and five substitutions at the following positions, 296,367, 440 and 563 of Cry1Ac1 holotype sequence (Figure 3).

## DISCUSSION

Indiscriminate use of broad-spectrum chemical insecticides has caused adverse effects to human health, other non-target organisms and has led to the build-up of chemical resistance in insect pests (Waage, 1997). Therefore, the urgent need for environmentally safe pest control is required to maintain sustainability of the environment. Deployment of integrated pest management (IPM) strategies to minimize crop production losses incurred due to insect pests can make significant contribution to food security in the developing countries.

The diversity of Bt strains facilitates isolation of new types of *cry* genes. PCR is a useful technique for quick and simultaneous screening of Bt strains for classification and prediction of insecticidal activities. Several screening projects of Bt collections from different parts of the world have been described (Juarez-Perez et al., 1997; Bravo et al., 1998; Ben-Dov et al., 1997; Thammasittirong and Attathom, 2008). In the present study, nine indigenous isolates of Bt were screened for the presence of *cry1* genes through PCR with the *cry1* family primers described by Juarez-Perez et al. (1997). Among the nine new isolates, only two showed amplification of expected size as in the case of the reference strain of Bt, 14R1 which is known to harbor for *cry1* genes.

Most of the commercial Bt formulations used for the

control of lepidopteran pests, contain toxins of Cry1A family, especially Cry1Aa, Cry1Ab and Cry1Ac proteins (Hofte and Whiteley, 1989). Cloning of the first *cry* gene, namely *cry1A(a),* was reported by Schnepf and Whiteley (1981). Recently, Xue et al. (2008) reported cloning of novel *cry1Ah* gene and its protein was more toxic to lepidopteran Asian corn borer. Swiecicka et al. (2008) reported that the novel Cry1Ab21 that produces a quasicuboidal crystal protein which is toxic to larvae of *Trichoplusia ni.*

In the present study, the partial *cry1* gene fragments amplified from genomic DNA of Bt strain, T133 are cloned in T/A vector. Nucleotide sequence of partial *cry1Ac* gene showed 98% similarity to the already reported *cry1Ac1* holotype sequence. The deduced amino acid sequences of partial Cry1Ac of Bt isolate, T133 showed difference at six positions in comparison to that of Cry1Ac1.

Result for the present study revealed the presence of new variant of *cry1Ac* in Bt isolate, T133. Variation of a single amino acid can significantly influence the level of toxicity in Cry proteins (Udayasuriyan et al., 1994; Liao et al., 2002). Therefore, further studies on expression of complete ORF of the novel *cry1Ac* cloned from the new isolate of Bt, T133 will be useful to know the insecticidal potency of its proteins.

## Conflict of Interests

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

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African Journal of Biotechnology

*Full Length Research Paper*

# Assessment of the genetic diversity of African yam bean *(Sphenostylis stenocarpa* Hochst ex. A Rich. Harms) accessions using amplified fragment length polymorphism (AFLP) markers

**Omena B. Ojuederie[1,2]*, Morufat O. Balogun[2], Iyiola Fawole[3], David O. Igwe[4] and Mercy O. Olowolafe[5]**

[1] Department of Biotechnology, Bells University of Technology, Ota, Nigeria.
[2]Department of Crop Protection and Environmental Biology, University of Ibadan, Ibadan Nigeria.
[3]Department of Biological Sciences, Bells University of Technology, Ota, Nigeria.
[4]Molecular Virology and Diagnostic Laboratory, International Institute of Tropical Agriculture, Ibadan Nigeria.
[5]Department of Biotechnology, Institute of Agricultural Research and Training, Moor Plantation, Ibadan, Nigeria.

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**The genetic diversity of 40 African yam bean (AYB) accessions was assessed using amplified fragment length polymorphism (AFLP) markers. Seeds of 40 accessions of AYB obtained from the International Institute of Tropical Agriculture (IITA) and Institute of Agricultural Research and Training (IAR&T) Ibadan, Nigeria, were grown in a greenhouse and young leaves from two weeks old plants collected for DNA extraction. The four primer combinations used generated a total of 1730 amplification fragments across the AYB accessions used in this study of which 1647 were polymorphic (95.20%). The number of amplified polymorphic AFLP bands per primer pair varied from 360 to 520 with an average percentage polymorphism of 95.6%. E-AGC/M-CAG produced the highest number of polymorphic bands (520). Polymorphic information content (PIC) values ranged from 0.9447 to 0.9626. The highest level of polymorphism (100%) was recorded for two primer combinations (E-AAC/M-CAG and E-ACT/M-CAG). The results of cluster analysis using UPGMA tree, grouped the 40 accessions of AYB into two major clusters with an overall similarity of 67.5%. The level of similarity between the accessions spanned 0.66 to 0.91. TSs 138 and TSs 139 were the most closely related accessions with high level of similarity index (0.91). Comparable results were obtained using Factorial Coordinate Analysis (FCO). The results from the present study confirm the robustness and the suitability of the AFLP as a molecular tool for the assessment of genetic diversity in AYB accessions.**

**Key words:** Amplified fragment length polymorphism (AFLP), cluster analysis, genetic diversity, *Sphenostylis stenocarpa,* polymorphism.

## INTRODUCTION

African yam bean (AYB) *Sphenostylis stenocarpa*, (Hochst. Ex. A. Rich) Harms is an important underutilized food crop in tropical Africa, the most economically important species in the genus *Sphenostylis* (Potter, 1992). It is an inexpensive source of protein (Apata and Ologhobo, 1990), high in sulphur containing amino acid

(Ezueh, 1977). The amino acid content in AYB seeds is higher than those in pigeon pea, cowpea, and bambara groundnut (Uguru and Madukaife, 2001, Adewale and Dumet, 2009). Despite the nutritional benefits of AYB, it is faced with several production constraints. The presence of high antinutritional factors such as tannins, saponins, oxalate, phytate, trypsin inhibitors and lectin, long cooking time, (Fasoyiro et al*.,* 2006) and low seed yield (Saka et al., 2004) are the major constraints to its cultivation and utilization. The current germplasm base available to breeders for genetic improvement is also narrow. AYB is among the 400 under exploited legumenous crops of the lowland tropics (Rachie and Robert, 1974) and is currently susceptible to genetic erosion, which would undoubtedly reduce the genetic bases and ultimately in the future, it is in danger of extinction (Klu et al., 2001). Utilization of underutilized crops such as AYB could be one of the best strategies to buffer nutritional, environmental and financial vulnerability (Jaenicke and Pasiecznik, 2009).

In order to improve any crop species with valuable genetic attributes by either plant breeders or scientists, germplasm characterization and assessment of genetic diversity are essential prerequisites. The degree of success in the genetic improvement of any crop species to a large extent depends on the amount of genetic diversity existing among cultivated accessions as well as their wild relatives. Such variations would make up a valuable source of potential parents for hybridization and subsequent development of improved cultivars. Cultivars with a great amount of diversity, if collected and appropriately evaluated would provide a great chance for breeders to select and disseminate best performing genotypes (Bainiwal and Jatasra, 1980). Most AYB accessions are in the hands of farmers and little activity has been carried out so far to genetically characterize these accessions. Only a very small sector of the farmers appreciates its cultivation, hence, they are the holder of the crop's genetic resources (Adewale et al., 2012). Explorations to collect more landraces from farmers are necessary to increase the gene pool for crop improvement. The Genetic Resources Centre of the International Institute of Tropical Agriculture only conserves a few accessions. Research on understanding the genetic diversity of AYB accessions through morphological characterization has been reported (Akande, 2008; Popoola et al., 2011; Adewale et al., 2012; Aremu and Ibirinde, 2012). Accessions are clustered into distinct groups based on the morphological data. Characterizations of morphological traits are however, affected by environmental factors and need to be assessed at particular stages of growth of the crop. It also requires expertise on crop and/or species.

AYB is one of the underutilized crops with a wide genetic base. So far only few studies have been carried out to assess the genetic diversity of AYB using molecular markers. Moyib et al. (2008) used random amplified polymorphic DNA (RAPD) markers to understand the pattern of genetic variability in a few Nigerian AYB accessions. Eleven RAPD primers were used for PCR amplification, but only nine of them produced scorable bands. The results of the study revealed high genetic diversity among accessions of AYB. Adewale (2011) assessed the genetic diversity in 80 AYB accessions using amplified fragment length polymorphism (AFLP) markers with five primer combinations. Very few bands were generated (227) of which only 59 were polymorphic. He obtained an average percentage polymorphism of 25.6% which is extremely low because majority of the bands were monomorphic. The potential of AFLP marker to detect high level of polymorphisms by single reaction has made AFLP as one of the popular molecular tools in understanding the pattern of genetic variability (Vos et al., 1995). AFLP technique can be used to assess genetic variations within a species or among closely related species.

Therefore, the major objective of this research is to assess the genetic diversity in forty (40) accessions of AYB mainly collected from different regions of Nigeria using AFLP molecular markers.

## MATERIALS AND METHODS

### Plant material

27 accessions of AYB were obtained from the Genetic Resources Center of the International Institute of Tropical Agriculture (IITA) Ibadan which consists of 2 accessions from Ghana, 1 from Bangladesh and 24 from Nigeria, with 8 of them collected from Enugu State in South East Nigeria, while 13 accessions were from the Institute of Agricultural Research and Training (IAR&T) Obafemi Awolowo University, Moor plantation Ibadan, all of Nigerian origin (Table 1). Most of the accessions from IAR&T were collected from South West Nigeria except AYB 34, which is from Kaduna State in North West Nigeria (Figure 1). Seeds of each accession were grown in the greenhouse and young leaves from two weeks old plants were collected for DNA extraction.

### DNA extraction

DNA samples were extracted from fresh leaf samples using a ZYMO ZR plant/seed DNA Miniprep extraction kit No. D6020

*Corresponding author. E–mail: omenabernojus@gmail.com. Tel : 2348075927894 or 2348185183876.



**Abbreviation: AFLP**, Amplified fragment length polymorphism; **AYB**, African yam bean; **FCO,** factorial coordinate analysis**; NUS**, neglected underutilized species; **PIC**, polymorphic information content; **TSs**, tropical *sphenostylis stenocarpa*; **UPGMA**, unweighted paired group method using arithmetic average.

**Table 1.** List of passport data of accessions used in this study.

| Accession Code | Country | Region | Collection site | Collector Name |
|---|---|---|---|---|
| TSs 5 | Nigeria | NA | NA | C.N. Aniagu |
| TSs 19 | Nigeria | NA | NA | Kanti Rawal |
| TSs 26 | Nigeria | NA | NA | Kanti Rawal |
| TSs 41 | Nigeria | NA | NA | Kanti Rawal |
| TSs 42 | Nigeria | NA | NA | Kanti Rawal |
| TSs 45 | Nigeria | NA | NA | Kanti Rawal |
| TSs 51 | Nigeria | NA | NA | L. Igbokwe |
| TSs 52 | Nigeria | NA | NA | L. Igbokwe |
| TSs 66 | Bangladesh | NA | NA | Dr N. Haq |
| TSs 68 | Ghana | NA | NA | W.M. Steele |
| TSs 78 | Nigeria | NA | NA | Unknown |
| TSs 88 | Nigeria | NA | NA | Badra |
| TSs 107 | Nigeria | NA | NA | Dr. N. Q. Ng |
| TSs 123 | Ghana | NA | NA | Eastwood/Holloway |
| TSs 133 | Nigeria | NA | NA | Unknown |
| TSs 134 | Nigeria | NA | NA | Unknown |
| TSs 137 | Nigeria | NA | NA | Unknown |
| TSs 138 | Nigeria | NA | NA | Unknown |
| TSs 139 | Nigeria | NA | NA | Unknown |
| TSs 140 | Nigeria | South East | Agbani | Dr. J. Machuka |
| TSs 148 | Nigeria | South East | Enugu | Dr. J. Machuka |
| TSs 150 | Nigeria | South East | Enugu | Dr. J. Machuka |
| TSs 152 | Nigeria | South East | Enugu | Dr. J. Machuka |
| TSs 153 | Nigeria | South East | Enugu | Dr. J. Machuka |
| TSs 154 | Nigeria | South East | Enugu | Dr. J. Machuka |
| TSs 156 | Nigeria | South East | Umueze | Dr. J. Machuka |
| TSs 157 | Nigeria | South East | Umueze | Dr. J. Machuka |
| AYB 1 | Nigeria | NA | NA | Dr (Mrs.) R. Akande |
| AYB 4 | Nigeria | South West | Ado-Ekiti | Dr (Mrs.) R. Akande |
| AYB 9 | Nigeria | NA | NA | Dr (Mrs.) R. Akande |
| AYB 23 | Nigeria | South West | Itaogbolu | Dr (Mrs.) R. Akande |
| AYB 26 | Nigeria | South West | Ilesha | Dr (Mrs.) R. Akande |
| AYB 34 | Nigeria | North West | Kaduna | Dr (Mrs.) R. Akande |
| AYB 45 | Nigeria | South West | Itaogbolu | Dr (Mrs.) R. Akande |
| AYB 50 | Nigeria | South West | Omi-Adio | Dr (Mrs.) R. Akande |
| AYB 56 | Nigeria | South West | Itaogbolu | Dr (Mrs.) R. Akande |
| AYB 57 | Nigeria | South West | Akure | Dr (Mrs.) R. Akande |
| AYB 61 | Nigeria | South West | Serafu | Dr (Mrs.) R. Akande |
| AYB 70B | Nigeria | NA | NA | Dr (Mrs.) R. Akande |
| AYB IFE | Nigeria | South West | Ife | Dr (Mrs.) R. Akande |

**TSs**, Tropical (*Sphenostylis stenocarpa*) obtained from the Genetic Resources Centre, International Institute of Tropical Agriculture, Ibadan; **AYB**, African yam beans obtained from the Institute of Agricultural Research and Training, Obafemi Awolowo University, Moor Plantation, Ibadan.

(Inqaba Biotech, South Africa) following the manufacturer's instructions. The qualities of the extracted DNA samples were checked using agarose gel electrophoresis (1.5%) stained with GR Green (Excellgen, Inqaba Biotechnical, South Africa). The DNA purity and integrity was assessed using Nanodrop spectrophotometer (ND-1000).

**Amplified Fragment Length Polymorphism (AFLP) analysis**

AFLP analysis was conducted according to Vos et al. (1995). Five AFLP primer combinations were screened and four were selected for PCR amplification (Table 2). Genomic DNA (100 ng/µl) were digested with 1 µL of EcoR1/MseI unit enzymes (Invitrogen AFLP

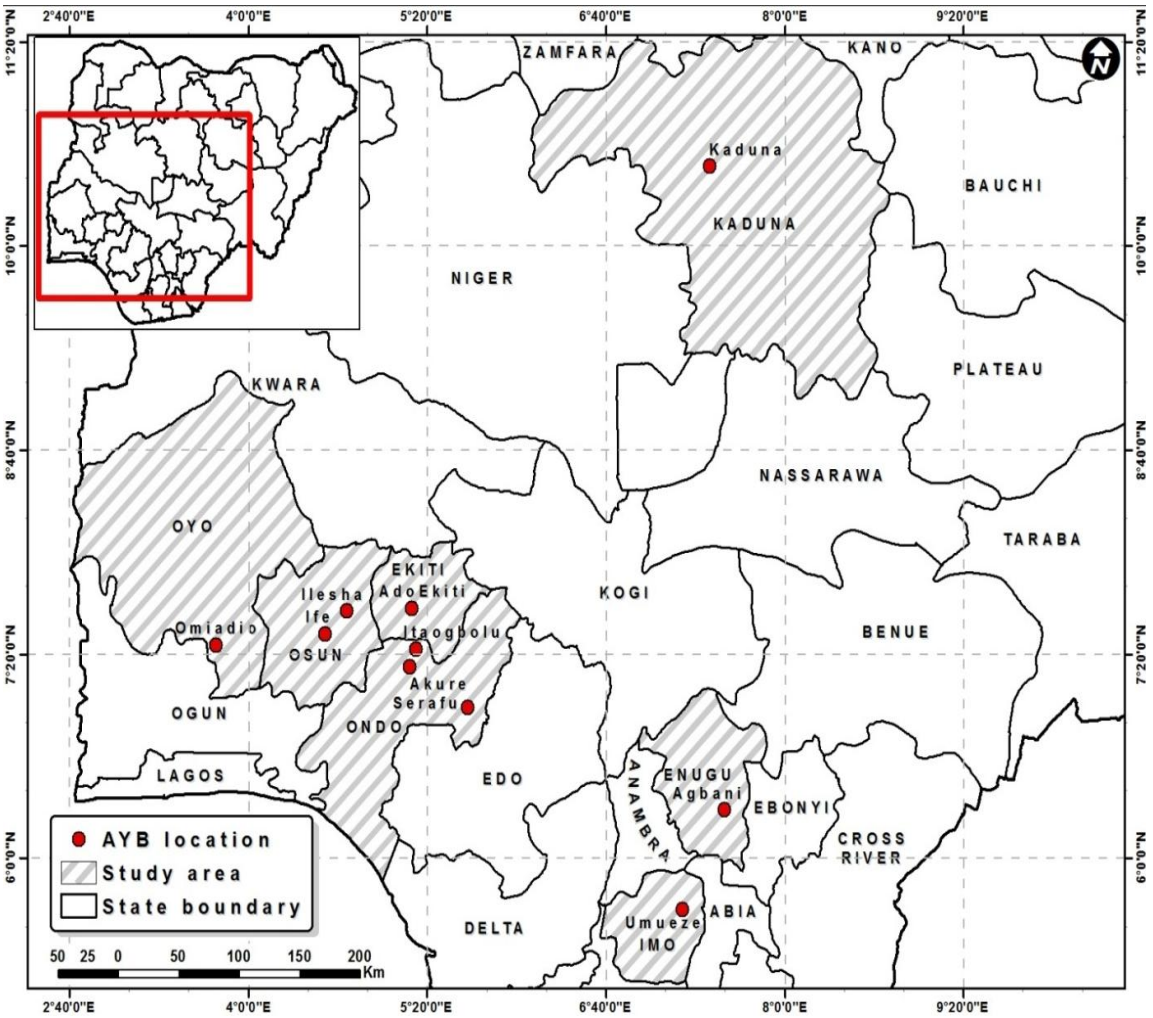


**Figure 1.** African yam bean accession collection sites in Nigeria.

**Table 2.** Adapters and primers used for pre-amplification and selective amplification of AFLP procedure.

| Name of Primer / adapter | Sequence (5'- 3') |
|---|---|
| ***EcoRI* adapter** | CTCGTAGACTGCGTACC<br>CATCTGACGCATGGTTAA |
| ***MseI* adapter** | GACGATGAGTCCTGAG<br>TACTCAGGACTCAT |
| ***EcoRI* primer** | |
| E- AAC | GACTGCGTACCAATTCAAC |
| E-ACG | GACTGCGTACCAATTCACG |
| E-ACT | GACTGCGTACCAATTCACT |
| ***MseI* primer** | |
| M-CAT | GATGAGTCCTGAGTAACAT |
| M-CAG | GATGAGTCCTGAGTAACAG |
| M-CTG | GATGAGTCCTGAGTAACTG |

Core reagent kit) incubated at 37ºC for 5 h after which the enzymes were inactivated at 70°C for 15 min. Pre selective amplification was carried out by ligating digested fragments with adapters following manufacturer's instruction. 10 µL of ligation reaction master mix was added to each of the tubes containing the digested genomic DNAs. PCR amplification was carried out using diluted ligations (1:10) mix. The PCR program in the thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, USA) was denatured for 2 min at 94°C, annealing at 56°C for 60 s and extension at 72°C for 60 s for a total of 25 cycles. Pre-selective PCR products were stored at 4°C. For selective amplification, pre-selective products were diluted (1:50) and then selective amplification was run using a touchdown programme {94°C for 30 min, 94°C for 30 s, 65°C for 30 s (-0.7 per cycle), 72°C for 60 s for a total of 12 cycles; 94°C for 30 s, 56°C for 30 s and 72°C for 60 s and 72°C for 2 min for a total of 23 cycles}.

**Data collection and Analysis**

Amplified DNA fragments were separated on a 6% denaturing polyacrylamide gel. The presence/absence of unequivocally scorable bands was transformed into a binary character matrix (1 for presence and 0 for absence of a band at a particular position). For cluster analysis, pair wise distance matrices were compiled by the NTYSYSpc 2.1 software packages (Rohlf, 2000), using the

**Table 3**. Polymorphism obtained from four primer combinations on 40 AYB accessions.

| Primer combination | Number of band | Number of monomorphic bands | Number of polymorphic band | Percentage of polymorphism (%) | PIC |
|---|---|---|---|---|---|
| E-AAC/M-CAG | 360 | 0 | 360 | 100 | 0.9447 |
| E-ACT/M-CAG | 383 | 0 | 383 | 100 | 0.9579 |
| E-AGC/M-CAG | 521 | 1 | 520 | 99.8 | 0.9573 |
| E-ACG/M-CAT | 466 | 82 | 384 | 82.4 | 0.9626 |
| Total | 1730 | 83 | 1647 | 382.2 | 3.8225 |
| Average | 432.5 | 20.75 | 411.75 | 95.55 | 0.9556 |

PIC, Polymorphic information content.

Jaccard's similarity coefficient (Jaccard, 1908). A dendrogram was constructed by unweighted paired group method using arithmetic average (UPGMA). For Factorial Coordinate Analysis (FCO) DARwin software package Version 5.0.158 (Perrier and Jacquemoud-Collet, 2006) was used. Polymorphic information content (PIC) was also calculated using the method described by Anderson et al. (1993) Thus:

$PIC = 1- \sum p_i^2$

Where; $p_i$ is the frequency of the $i^{th}$ allele.

## RESULTS

### Polymorphism as detected by amplified Fragment Length Polymorphisms (AFLPs)

The four informative primer combinations generated a total of 1730 amplification fragments across all AYB accessions of which 1647 were polymorphic (95.20%). The number of amplified polymorphic AFLP bands per primer pair varied from 360 to 520 with an average of 411.75. An average percentage polymorphism of 95.6% was also generated. E-AGC/M-CAG produced the highest number of polymorphic bands 520, followed by E-ACG/M-CAT (384), E-ACT/M-CAG (383 bands), and E-AAC/M-CAG (360). Primer combination E-ACG/M-CAT produced the highest number of monomorphic bands (82) (Table 3). Primer combinations E-AAC/M-CAG and E-ACT/M-CAG displayed the highest percentage polymorphism (100%). Polymorphic information content (PIC) values ranged from 0.9447 to 0.9626.

### Cluster analysis of AYB accessions

The dendrogram grouped the accessions into two major clusters with an overall similarity of 67.5% (Figure 2). The level of similarity between the accessions ranged from 0.66 to 0.91. TSs 138 and TSs 139 were the most closely related accessions with a similarity index of 0.91. Cluster 1 was further grouped into two sub clusters 1.1 and 1.2. Twenty seven accessions were found to cluster together in sub cluster 1.1, while six accessions were grouped in sub cluster 1.2. Out of these six accessions, a pair of accessions; TSs 68 (from Ghana) and TSs 66 (from Bangladesh), and TSs 107 and TSs 88 (both from Nigeria) were found to be genetically close to each other compared to TSs 78 and TSs 123 which displayed some degree of genetic distinctness. The pattern of clustering of accessions appears to be based on their geographical location. Thus, for example, some of the accessions that cluster together in sub cluster 1.1 were from South West Nigeria (AYB 4, Ado-Ekiti (Ekiti State), AYB 50, Omi-adio (Oyo State), AYB 26, Ilesha (Osun State), AYB IFE, Ife (Osun State) and AYB 57, Akure; AYB 23, AYB 45 and AYB 56, Itaogbolu (Ondo State)) and some were also from South East Nigeria (TSs 140, TSs 148, TSs 150, TSs 152, TSs 153, TSs 154, all from Enugu State and TSs 156 and TSs 157 from Imo State). In Cluster II, two sub clusters were identified one containing six accessions (TSs 19, TSs 26, TSs 41, TSs 42, TSs 51 and TSs 52) while the other only with a single accession TSs 45.

### Factorial coordinates analysis of 40 African yam bean accessions

Factorial coordinate analysis generated by DARwin software version 5.0 using dissimilarity coefficient matrix placed the 40 AYB accessions into four groups (Figure 3). Group 1 includes four accessions (TSs 134, TSs137, TSs 138 and TSs 139). These accessions also appeared as a subgroup in sub cluster 1.1 of the dendrogram. Group II contain eight accessions: TSs 140, TSs 148, TSs 150, TSs 152, TSs 153, TSs 154 (Enugu State) and TSs 156 and TSs 157 (Imo State) in South East Nigeria. These accessions were also clustered together in sub cluster 1.1 on the dendrogram (Figure 2). The accessions in group III belong to collections from South West Nigeria which was obtained from the Institute of Agricultural Research and Training (IAR&T) Ibadan. These accessions include AYB 1, AYB 4, AYB 9, AYB 23, AYB 26, AYB 34, AYB 45, AYB 50, AYB 56, AYB 57, AYB 61, AYB 70B and AYB IFE. In this group, AYB 23, AYB 26 and AYB 34 were more closely placed on the scatter plot. This was also reflected on the dendrogram in sub cluster 1.2 with AYB 23 and AYB 26 closely related

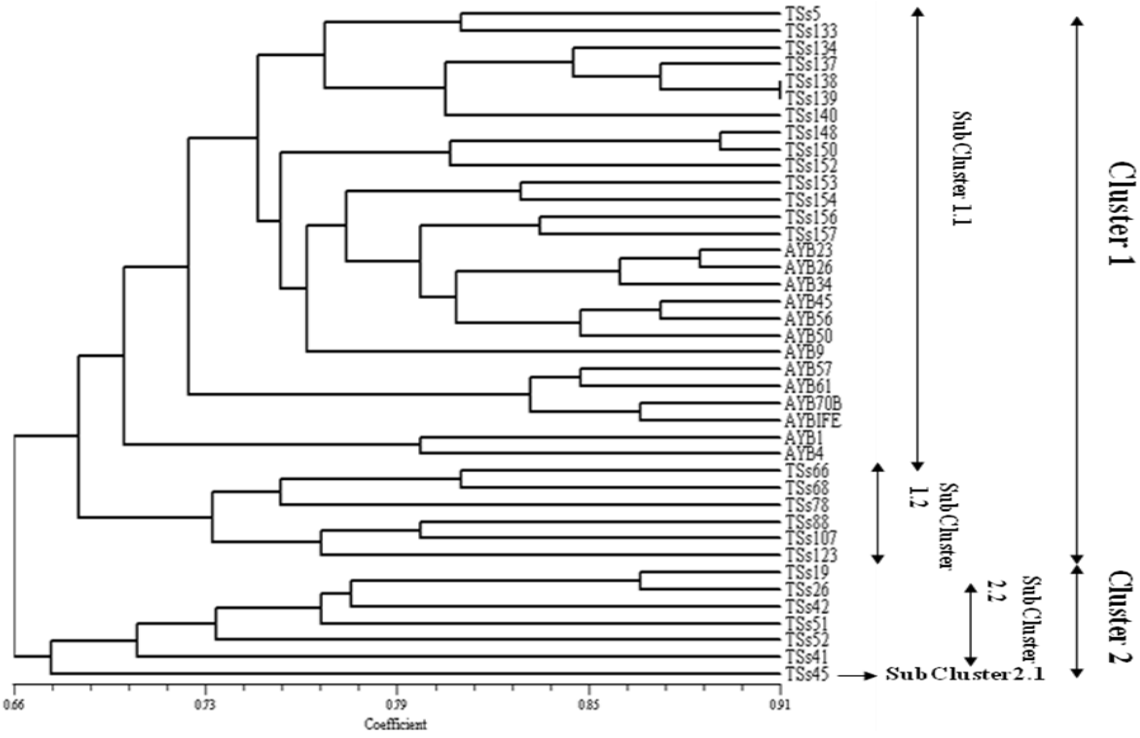


**Figure 2.** Dendrogram of 40 African yam bean accessions revealed by UPGMA cluster analysis using Jaccard's similarity coefficient.

and AYB 34 which was collected from Kaduna (North West Nigeria) displayed some degree of genetic isolation (Figure 2). The other fifteen accessions were found to be included in the 4th group. The results of both cluster and factorial coordinate analysis showed some resemblance in the pattern of grouping the 40 AYB accessions studied.

## DISCUSSION

The present study revealed the extent and organization of genetic diversity within 37 selected accessions of AYB from Nigeria, 1 from Bangladesh and 2 from Ghana assessed with AFLP markers. High levels of genetic variation were observed. Both cluster and FCO tend to group the accessions based on their geographic origin and this might indicate considerable genetic divergence between the accessions reflecting different growing environments. TSs 68 and TSs 123 both from Ghana were clustered in sub cluster 1.2 along with three Nigerian accessions TSs 88 and TSs 107 which showed close resemblance and TSs 78 with some level of genetic distinctness just as TSs 123. TSs 66 from Bangladesh was closely linked with TSs 68; it may have originated from Ghana. From the dendrogram, AYB 1 was found to be closely related to AYB 4. This could suggest that this accession could also be collected from Ado-Ekiti (Ekiti State), South West Nigeria. The same is true for AYB 70B which was closely related to AYB IFE collected from Ile- Ife in Osun State. The level of polymorphisms ranged from 82.4% (E-ACG/M-CAT) to 100% (E-ACT/M-CAG and E-AAC/M-CAG). TSs 148, TSs 150, TSs 152, TSs 153, and TSs 154 originated from Enugu (Enugu State South East Nigeria) hence their grouping in sub cluster 1.1. TSs156 and TSs 157 both from Umueze (Imo State, South East Nigeria), showed close resemblance.

The accessions from Enugu were closely linked in group II of the scatter plot (Figure 3) while the two accessions from Umueze TSs156 and TSs 157 and TSs 140 from Agbani were clearly distinct. The FCO placed the accessions with known passport data into two geographically distinct regions South East (Group II) and (Group III) South West Nigeria. Accessions in Groups IV may have been from South South Nigeria where AYB is also widely cultivated and utilized.

Estimates of genetic distances based on differences in AFLP patterns are informative about genetic diversity (Greef et al*.,* 1997; Powell et al., 1996), phylogeny (Hill et al., 1996) and the geographic origins of genotypes and gene pools of plants (Beismann et al., 1997, Paul et al., 1997).

Moyib et al. (2008) also reported considerable genetic diversity among 24 AYB accessions of Nigerian origin from the Department of Agronomy, University of Ibadan, Nigeria, using random amplified polymorphic DNA (RAPD) markers which clustered the accessions into 8

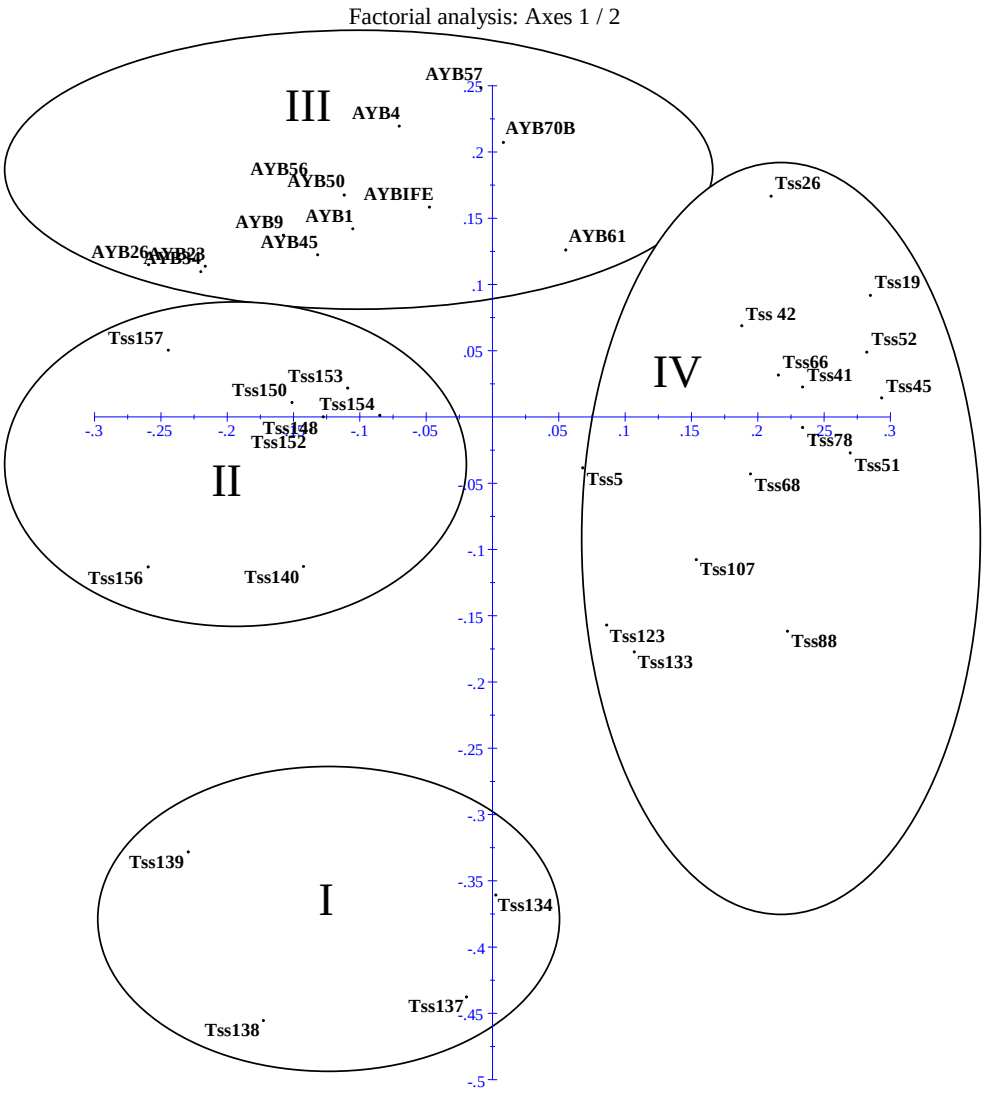


**Figure 3.** Factorial coordinate analysis for African yam bean accessions as generated by DARwin software version 5.0.0.158 using similarity coefficient matrix.

distinct cluster groups at 80% similarity index. The similarity indices ranged from 0.42 to 0.96 which were considered to be useful in facilitating the development of large number of new varieties through hybridization and transfer of useful genes, thus maximizing the use of such available germplasm as genetic resource materials for breeders.

In the work of Adewale (2011), 80 AYB accessions were characterized using five AFLP primer combinations (E-AAC/M-CAG, E-ACT/M-CAG, E-AGC/M-CAG, E-ACG/M-CAT and E-ACG/M-CTG) which generated a low percentage polymorphism of 26% due to the many monomorphic fragments obtained (168). This revealed higher similarities across some of the genomic loci of the tested population of AYB, an indication of low genetic diversity among the collected accessions by Adewale (2011). In the present study using four of the primer combinations, E-AAC/M-CAG, E-AGC/M-CAG, E-ACT/M-CAG and E-ACG/M-CTG, a higher average polymorphic content of 0.96 and percentage polymorphism of 95.6% were obtained which indicates a higher genetic diversity across the genomic loci of the 40 AYB accessions studied. The study also revealed that the 37 AYB accessions collected from different places in Nigeria represent a genetically diverse population.

Genetic fingerprinting using AFLP markers has been reported in various crops; Dolichos Bean (Venkatesha et al. 2010); Velvet bean (Capo-chichi et al. 2003); and in assessment of genetic diversity in sesame (*Sesamum indicum* L.) (Ali et al. 2007). It has also been used successfully for analyzing genetic diversity in some other plant species such as peanut (Herselman, 2003) and soy-

bean (Ude et al. 2003). In these studies different primer combinations were used to assess genetic diversity. The AFLP primer combinations used for AYB in the present study are efficient for genetic diversity studies in the species. AFLP markers are the most ideal genetic markers for analysis of genetic diversity both at intraspecific and interspecific level particularly for closely related species and underutilized crops.

For selection of good parental material for heterosis breeding program, the diversity results obtained through AFLP can be used to correlate with the pedigree relationship and morphological traits for genetic improvement in AYB. Having a good understanding of the degree of genetic relationships among AYB accessions will be of paramount importance for genetic improvement of the crop.

## Conclusion

The results obtained from the present study showed the robustness and the suitability of AFLP as a molecular tool for diversity analysis and for the assessment of genetic diversity among individuals of AYB species. The high percentage polymorphism obtained (95.6%), indicates the heterogeneity of the AYB accessions used. Primer combinations E-ACT/M-CAG and E-AAC/M-CAG which produced 100% level of polymorphism are the most useful primer combinations for diversity analysis of AYB accessions. The genetic relatedness among the accessions could provide useful information regarding selecting both potential cross parents and desirable phenotypic traits for breeding programs.

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## Conflict of Interests

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

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# Microbial composition of guava (*Psidium guajava*), hibiscus (*Hibiscus-rosa sinensis*), mango (*Mangifera indica*) and pumpkin (*Telfairia occidentalis* Hook) phyllosphere

**C.B. Chikere* and C.C. Azubuike**

Department of Microbiology, University of Port Harcourt, P.M.B. 5323, Port Harcourt, Rivers State, Nigeria.

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**Diverse groups of microorganisms colonize phyllosphere and carry out different but specific ecological functions. The phyllosphere of four different plants; guava (*Psidium guajava*), hibiscus (*Hibiscus-rosa sinensis*), mango (*Mangifera indica*) and pumpkin (*Telfairia occidentalis Hook*), each from four different sites Aluu Community Health Centre, Lulu Briggs Health Centre, University of Port Harcourt Abuja campus, and University of Port Harcourt Teaching Hospital were examined for bacterial and fungal growth using culture-dependent technique. A total of 32 bacterial and 13 fungal species covering seven and five genera respectively were isolated and characterized as *Bacillus*, *Enterobacter, Escherichia coli, Pseudomonas, Salmonella, Shigella* and *Staphylococcus; Aspergillus*, *Fusarium*, *Mucor*, *Penicillum* and *Rhizopus*. Among the epiphytes, bacteria were found to predominate. Statistically, plant type and sampling location were found to extremely influence observed microbial composition associated with each plant and site at P<0.0001. The microbial genera isolated from this study showed that, both human and plant pathogens can colonize plants' phyllosphere. Since most of the edible leafy vegetables have less waxy phyllosphere which permit microbial growth, it is necessary that they be washed and cooked properly before consumption to avoid ingestion of possible food pathogens that cause food-borne diseases.**

**Key words:** Epiphytes, location, pathogens, plant type.

## INTRODUCTION

Microorganisms are known to be ubiquitous, their growth and survival depend on complex sets of environmental and nutritional factors. The ubiquity of microorganisms is supported by Baas-Becking hypothesis that: "everything is everywhere but the environment selects" (de Wit and Bouvier, 2006). With reference to plants, there are three major microhabitats where microorganisms can inhabit depending on the one that most supports their growth (Montesino, 2003). These microhabitats are the aerial plant part (the phyllosphere), the zone of influence of root system (the rhizosphere) and the internal transport system (the endosphere). Microorganisms which inhabit

*Corresponding author. E-mail: chioma.chikere@uniport.edu.ng. Tel: 07030912861.

such microhabitats are called epiphytes, rhizophytes and endophytes respectively (Montesino, 2003). Phyllosphere therefore is a microhabitat on the surface of plant's leaf where diverse group of microorganisms colonize and carry out their different and specific ecological function. The diversity of the microbial composition of phyllosphere include algae, bacteria, filamentous fungi, yeast and in rare cases nematodes and protozoans (Morris et al., 2002; Lindow and Brandl, 2003) with bacteria being the most abundant among the diverse groups of phyllosphere microorganisms (Inacio et al., 2002; Mukhtar et al., 2010). There are several sources to which microbial colonization of phyllosphere can be traced and they include animals, rain drops, wind, and other agents of cross and self pollination.

Suslow (2002), classified phyllosphere microorganisms into two major groups, transient epiphytes which show no limitation to multiplication and residual epiphytes which have the capacity to multiply in the absence of wounds. Microorganisms on the phyllosphere may occur individually but usually they occur as aggregate or biofilm-like structures containing bacteria, yeast and filamentous fungi (Bélanger and Avis, 2000; Jacques et al., 2005). Bacteria and fungi are known to be friends and foes of human, their specific role as friends on phyllosphere has little effect compared to the foes activities which they may impact on human upon consumption.

The study was aimed at determining the different bacteria and fungi associated with some plants' phyllosphere using culture-dependent technique and to compare the effect of sample sites on the extent of phyllosphere colonization by epiphytes.

## MATERIALS AND METHODS

### Sampling

The phyllosphere of four different plants namely guava (*Psidium guajava*), hibiscus (*Hibiscus-rosa sinensis*), mango (*Mangifera indica*) and pumpkin (*Telfairia occidentalis Hook*) each from four different sites, University of Port Harcourt Teaching Hospital (T), University of Port Harcourt Health Centre Lulu Briggs (L), Aluu Community Health Centre (A) and University of Port Harcourt Abuja campus (U). The plants' leaves used for the study were identified by Mrs. Josephine Agogbua at the Department of Plant Science and Biotechnology Herbarium, University of Port Harcourt, Nigeria. The leaves surfaces were aseptically swabbed. After the collection of the samples, 0.5 ml of diluent (phosphate buffer saline) was added to the swab container and transported in sterile polyethylene bag to the Microbiology Laboratory, Department of Microbiology, University of Port Harcourt where bacteriological and mycological analyses were carried out. In other to ensure the isolation of autochthonous epiphytes, the samples were collected in the morning three days after the last rain fall (Yashiro et al., 2011).

### Isolation of bacteria and fungi

Appropriate aliquots of 5-fold serially diluted samples were aseptically transferred onto MacConkey agar, Mannitol salt agar, plate count agar and Salmonella-shigella agar plates (Yadav et al., 2010; Yashiro et al., 2011). To obtain discrete colonies and even distribution of colonies on the agar plates, the primary inocula were streaked in three directions after which the inoculated plates for bacterial isolation were incubated at 37°C for 48 h. Subculturing of the isolates was done to obtain pure colonies for biochemical tests. Discrete colonies of the bacteria to be identified were aseptically subcultured on nutrient agar medium. The various isolates were differentiated by their colonial morphologies (colour, edge, elevation, shape, size and texture) on the respective media after 24 h of incubation following the subculture. A tentative identification of bacterial isolates was made based on the results of various biochemical tests carried out (Cheesbrough, 2006). Fungal selective medium (Sabouraud dextrose agar, SDA) was prepared and supplemented with a known concentration of 0.05% (v/v) of chloramphenicol for fungal isolation. Following this, 1.0ml of the serially diluted samples were aseptically transferred onto the freshly prepared SDA, the plates were later incubated at 30°C for 7 days (Yadav et al., 2010; Yashiro et al., 2011). Fungal identification was done under microscope using fungal atlas after KOH mount and lactophenol cotton blue staining (Malloch, 1997).

### Statistical analysis

In order to determine the effects of sampling site and type of plant phyllosphere on the bacterial diversity observed, the data (frequency of isolation of plant-associated microbes) were subjected to statistical analysis using 2-way ANOVA and Bonferroni's multiple comparison test using GraphPad Prism software for Windows version 5.01.

## RESULTS

### Bacterial isolates from the phyllosphere samples

Culture-dependent technique was employed to study the diversity of microbial communities on the phyllosphere of four selected plants: *Hibiscus-rosa sinensis, Mangifera indica, Psidium guajava* and *Telfairia occidentalis* Hook. A total of 32 bacterial isolates covering seven genera were obtained and characterized as *Bacillus*, *Enterobacter, Escherichia, Pseudomonas, Salmonella, Shigella,* and *Staphylococcus.* It was observed that more bacteria (45.45%) were isolated from the phyllosphere of mango than other phyllosphere from the University of Port Harcourt. Also, no bacterium was isolated from the phyllosphere of guava from the same sample site (Table 1). More bacterial isolates (50%) were obtained from the phyllosphere of pumpkin from the University of Port Harcourt Teaching Hospital. In this site also, no bacterium was isolated from the phyllosphere of mango (Table 1). The bacteria isolated from the phyllosphere of pumpkin in Aluu Health Centre and Lulu Briggs Health Centre (50% and 42.89% respectively) predominated over the phyllosphere of other plants in both sites (Table 1). It was also observed that pumpkin had the highest frequency (43.75%) of bacterial isolates from the phyllosphere of the sampled plants, followed by hibiscus (25%), mango (18.75%) and guava (12.50%) (Figure 1). There was an even distribution of bacteria isolated from the phyllosphere of pumpkin and hibiscus, this contributed to them having the highest frequency of bacterial isolates,

**Table 1.** Bacterial isolates from different samples and sites.

| Isolate code | Shape | Gram reaction | Oxidase | Indole | MR | VP | Citrate | Motility | Catalase | Slant | Butt | $H_2S$ | Gas | Glucose | Lactose | Tentative Identity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **UM1** | R | - | + | - | - | - | + | + | + | A | A | - | + | + | + | *Pseudomonas* sp. |
| **UM2** | R | + | - | + | - | + | + | - | + | B | A | + | + | + | - | *Bacillus* sp. |
| **UM3** | R | - | - | + | + | - | + | + | + | B | A | + | + | + | + | *Salmonella* sp. |
| **UM4** | R | - | - | + | + | - | - | + | + | A | A | - | + | + | + | *Escherichia* sp. |
| **UM5** | C | + | - | - | - | - | + | - | + | A | A | - | - | + | + | *Staphylococcus* sp. |
| **UH1** | R | - | + | - | - | - | + | + | + | A | A | - | + | + | + | *Pseudomonas* sp. |
| **UH2** | C | + | - | - | - | - | + | - | + | A | A | - | - | + | + | *Staphylococcus* sp. |
| **UP1** | R | - | + | - | - | - | + | + | + | A | A | - | + | + | + | *Pseudomonas* sp. |
| **UP2** | R | + | - | + | - | + | + | - | + | B | A | + | + | + | - | *Bacillus* sp. |
| **UP3** | R | - | - | + | + | - | + | + | + | B | A | + | + | + | + | *Salmonella* sp. |
| **UP4** | R | - | - | + | + | - | - | + | + | A | A | - | + | + | + | *Escherichia* sp. |
| **TGI** | R | - | + | - | - | - | + | + | + | A | A | - | + | + | + | *Pseudomonas* sp. |
| **TH1** | R | - | + | - | - | - | + | + | + | A | A | - | + | + | + | *Pseudomonas* sp. |
| **TH2** | R | + | - | + | - | + | + | - | + | B | A | + | + | + | - | *Bacillus* sp. |
| **TH3** | C | + | - | - | - | - | + | - | + | A | A | - | - | + | + | *Staphylococcus* sp. |
| **TP1** | R | - | + | - | - | - | + | + | + | A | A | - | + | + | + | *Pseudomonas* sp. |
| **TP2** | R | - | - | - | + | - | - | - | - | B | A | - | - | + | - | *Shigella* sp. |
| **TP3** | R | - | - | - | - | + | + | + | + | A | A | - | + | + | + | *Enterobacter* sp. |
| **TP4** | C | + | - | - | - | - | + | - | + | A | A | - | - | + | + | *Staphylococcus* sp. |
| **AG1** | R | - | + | - | - | - | + | + | + | A | A | - | + | + | + | *Pseudomonas* sp. |
| **AG2** | R | - | - | + | + | - | - | + | + | A | A | - | + | + | + | *Escherichia* sp. |
| **AH1** | R | - | - | + | + | - | - | + | + | A | A | - | + | + | + | *Escherichia* sp. |
| **AP1** | R | + | - | + | - | + | + | - | + | B | A | + | + | + | - | *Bacillus* sp. |
| **AP2** | R | - | - | - | + | - | - | - | - | B | A | - | - | + | - | *Shigella* sp. |
| **AP3** | R | - | - | - | - | + | + | + | + | A | A | - | + | + | + | *Enterobacter* sp. |
| **LM1** | R | - | + | - | - | - | + | + | + | A | A | - | + | + | + | *Pseudomonas* sp. |
| **LG1** | R | - | + | - | - | - | + | + | + | A | A | - | + | + | + | *Pseudomonas* sp. |
| **LH1** | R | - | - | + | + | - | - | + | + | A | A | - | + | + | + | *Escherichia* sp. |
| **LH2** | C | + | - | - | - | - | + | - | + | A | A | - | - | + | + | *Staphylococcus* sp. |
| **LP1** | R | - | - | + | + | - | + | + | + | B | A | + | + | + | + | *Salmonella* sp. |
| **LP2** | R | - | - | - | + | - | - | - | - | B | A | - | - | + | - | *Shigella* sp. |
| **LP3** | R | - | - | + | + | - | - | + | + | A | A | - | + | + | + | *Escherichia* sp. |

Key: positive (+); negative (-); rod (R); cocci (C); acid (A); alkaline (B). Isolate code: the first letters represent the samples sites (A, L, T, and U), the second letters represent the samples guava (G), hibiscus (H), mango (M) and pumpkin (P), the Arabic numerals represent the isolate number.

unlike the phyllosphere of mango and guava from the University Teaching Hospital and University campus sites in which no bacterium was isolated (Table 1).

*Pseudomonas* sp. (28.13%) and *Escherichia* sp. (18.18%) were found to be the predominant bacteria among the isolates, while *Enterobacter* sp. (6.25%) was found to have the least frequency of occurrence among the bacteria isolated (Figure 2). From the foregoing, it was observed that Gram negative bacteria (71.88%) predominated over Gram positive bacteria (28.13%). Sampling location significantly influenced the types of bacterial species while the type of plant phyllosphere sampled was an extremely significant factor that also selected specific bacteria found in such plants at $P<0.0001$. Multiple comparison tests using Bonferroni indicated that plants phyllosphere sampled in University of Port Harcourt Abuja campus when compared with other locations showed very significant bacterial diversity

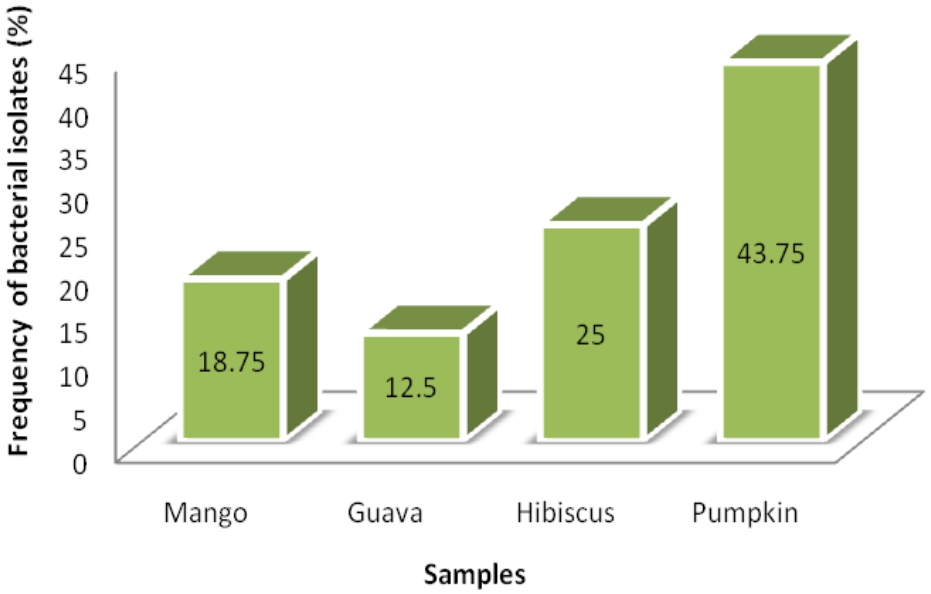


**Figure 1.** Frequency of bacterial isolates from different phyllosphere samples.

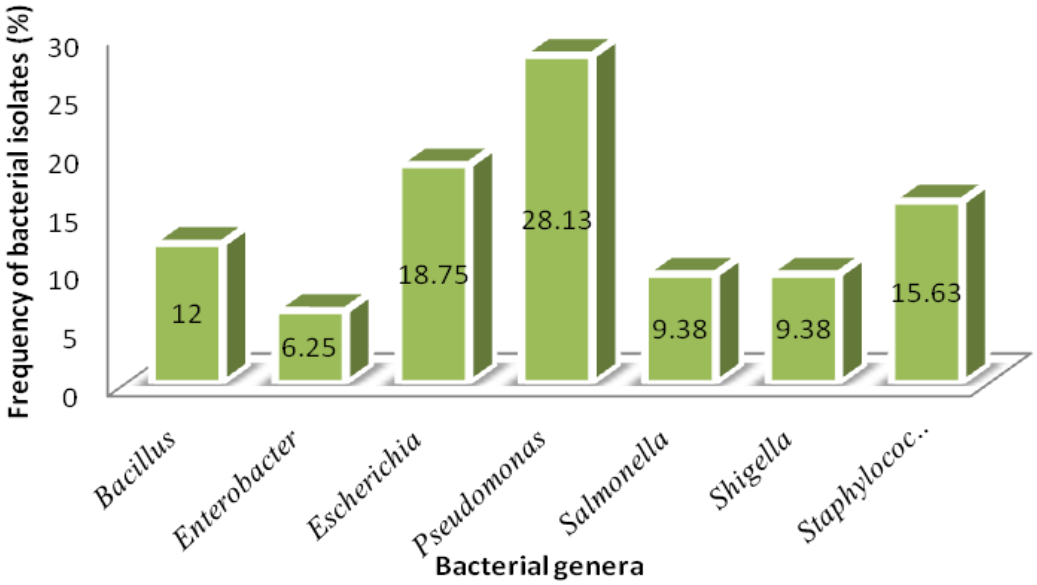


**Figure 2.** Frequency of bacterial genera from all the phyllosphere samples collected from the four sampled sites.

at P<0.001.

## Fungal isolates from the phyllosphere samples

Thirteen fungal species covering five genera were also isolated and characterized as *Aspergillus* (30.77%), *Fusarium* (7.69%), *Mucor* (30.77%), *Penicillum* (15.38%) and *Rhizopus* (15.38%). It was also observed that *Aspergillus* and *Mucor* were the predominant fungi from the phyllosphere of the four different plants (Table 2 and Figure 4). Fungal spp. were isolated from all the phyllosphere of pumpkin in the different sampled sites (Table 2) but no fungus was isolated from the phyllosphere of hibiscus for all the sampled sites. Also, no fungal isolate was obtained from the phyllosphere of mango and guava from Teaching Hospital and Lulu Briggs Health Centre sites (Table 2 and Figure 3).

**Table 2.** Fungal isolates from different samples and sites.

| Isolate code | Colony characteristics | Morphological characteristics | Tentative identity |
|---|---|---|---|
| Um1 | yellow-brown colonies | Double spores forming entire vesicle and radiate head at the end of a long erect conidiophores | *Aspergillus* sp. |
| Um2 | Green colonies | Coenocytic mass of mass branching hyphal growth with conidia lined at the tips of each branch of hyphae | *Penicillium* sp. |
| Ug1 | White-grey | Long conidiophores having single and double spores that cover the entire vesicle. | *Mucor* sp. |
| Up1 | White-brown | Long conidiophores having single double spore that cover the entire | *Aspergillus* sp. |
| Up2 | Grey colonies | Single-celled conidia. Coenocytic mass of simple branching hyphal growth with condia lined at the tip of each branch of hyphae. | *Rhizopus* sp. |
| Tp1 | White-brown | Non septate hyphae with branched long sporangiophores | *Mucor* sp |
| Tp2 | Pink –orange | Macroconidia and microconidia with two hyaline each | *Fusarium* sp. |
| Am1 | White-brown | Long conidiophores having single double spore which cover the entire vesicle | *Aspergillus* sp. |
| Ap1 | yellow-brown colonies | Double spores forming entire vesicle and radiate head at the end of a long erect conidiophores | *Aspergillus* sp. |
| Ap2 | Green colonies | Coenocytic mass of mass branching hyphal growth with conidia lined at the tips of each branch of hyphae | *Penicillium* sp. |
| Ap3 | White-brown | Non septate hyphae with brached long sporangiophores | *Mucor* sp |
| Lp1 | Grey colonies | Single-celled conidia. Coenocytic mass of simple branching hyphal growth with condia lined at the tip of each branch of hyphae. | *Rhizopus* sp. |
| Lp2 | White-grey | Long conidiophores having single and double spores which cover the entire vesicle. | *Mucor* sp. |

Isolate code: The first letters represent the samples sites (A, L, T, and U), the second letters represent the samples guava (G), hibiscus (H), mango (M) and pumpkin (P), the Arabic numerals represent the isolate number.

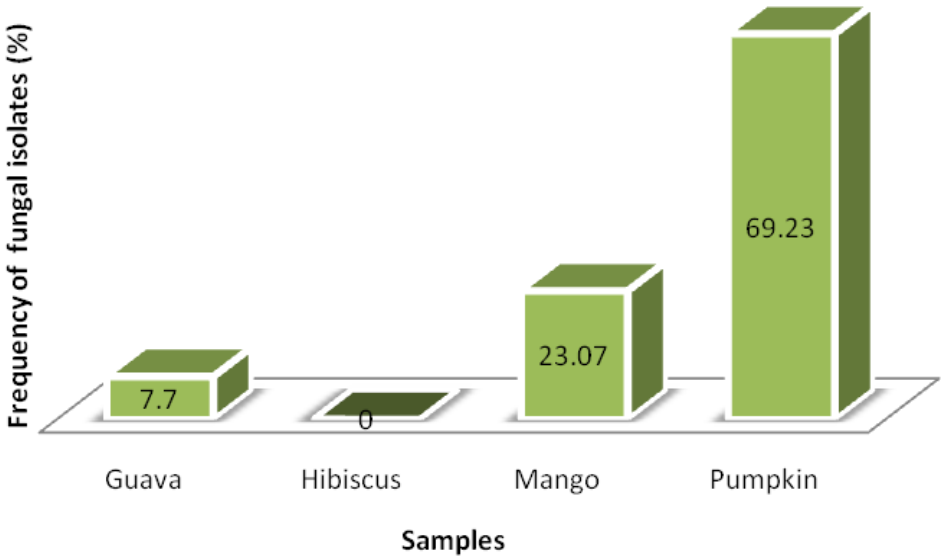


**Figure 3.** Frequency of fungal isolates from different phyllosphere samples.

Statistical analysis indicated that the type of plant phyllosphere and sampling location both greatly influenced the fungal diversity observed at P<0.0001. Post ANOVA test showed that plants phyllosphere in University of Port Harcourt Abuja campus as earlier observed had more fungal species when compared with other locations at P<0.001 (Figure 5).

## DISCUSSION

The highest percentage (43.75%) of bacteria obtained

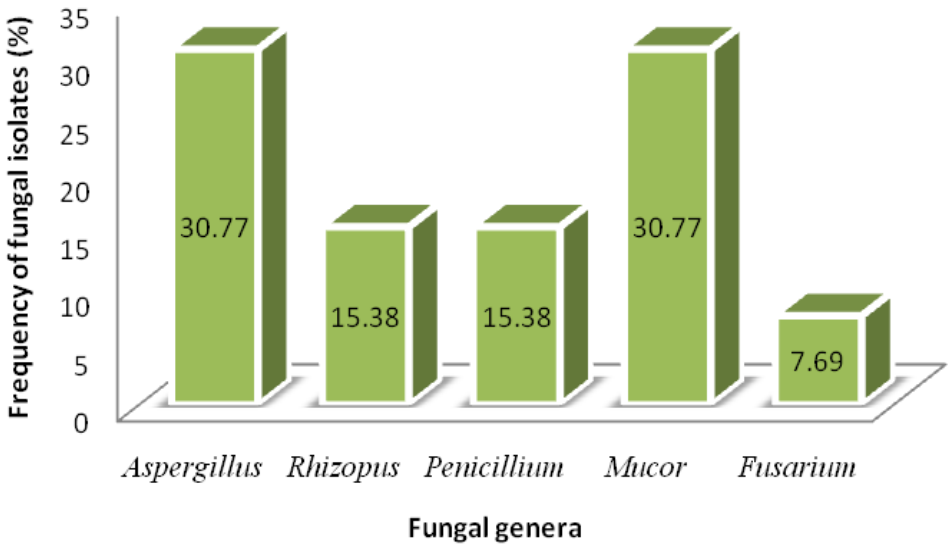


**Figure 4.** Frequency of fungal genera from all the phyllosphere samples collected from the four sampled sites.

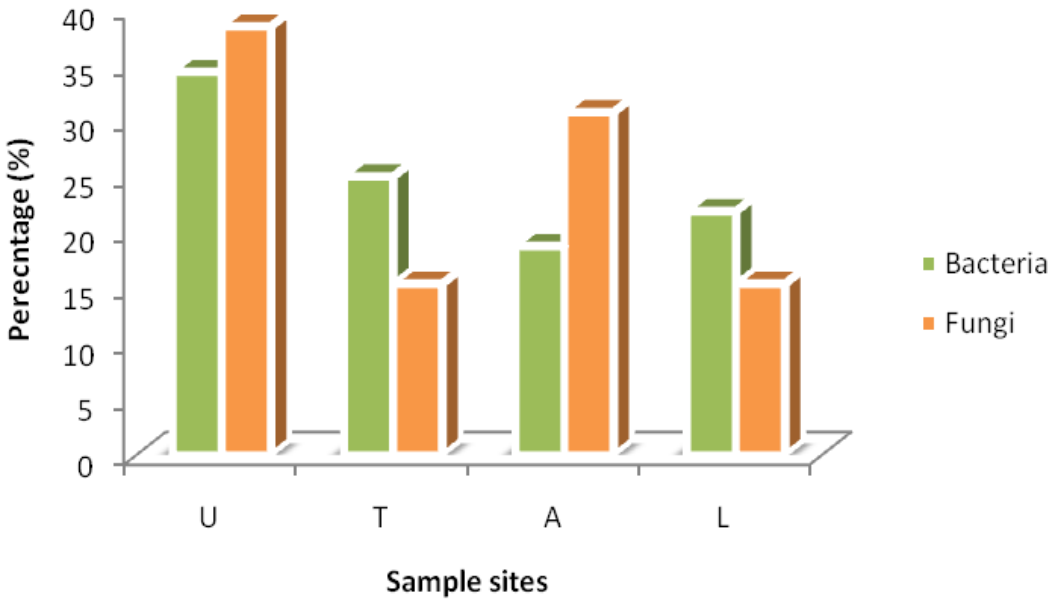


**Figure 5.** Percentage distribution of bacteria and fungi from the sample sites. U, University of Port Harcourt Abuja Campus; T, University of Port Harcourt Teaching Hospital; A, Aluu Community Health Centre and L, University of Port Harcourt Health Centre Lulu Briggs.

from the phyllosphere of pumpkin within the sampled sites was attributed to the plant species which appear to influence the microbial carrying capacity of the leaf. Pumpkin leaves are broad and less waxy; it is also located close to the ground surface compared to other leaves used in this study and tends to have more surface area for bacteria to thrive (Lindow and Brandl, 2003). Researchers have shown that, the total number of culturable bacteria recovered from broad-leaf plants were significantly greater than that recovered from grasses or waxy broad-leaf plant (Kinkel et al., 2000; Lindow and Brandl, 2003). The less frequency of bacteria obtained from hibiscus (25%), Mango (18.75%) and guava (12.5%) as compared to pumpkin was as a result of the waxy nature of the plant leaves (Kinkel et al., 2000; Lindow and Brandl, 2003). Waxy layer of plant leaf limit passive diffu-

sion of nutrient and water vapour from the plant interior onto the surface and defines the hydrophobicity of the leaf (Lindow and Brandl, 2003). Thick waxy cuticles have been implicated to interfere with bacteria colonization of plants by limiting diffusion of nutrient and inhabiting the wetting of the leaf surface (Kinkel et al., 2000; Knoll and Schreiber 2000; Beattie, 2002; Beattie and Marcell, 2002; Lindow and Brandl, 2003). The predominance of *Pseudomonas* sp. (28.13%) over other bacteria isolated can be explained on the bases that, Pseudomonas spp. have the ability to modify their microbial habitat in order to increase nutrient availability on the phylloplane (Lindow and Brandl, 2003). Also, some phyllosphere bacteria have been shown to increase the permeability of the cuticle enhancing water and nutrient availability (Schreiber et al., 2005) while some are able to increase their nutrient availability by producing toxins which affect ion transport across plant cell plasma membrane (Hirano and Upper, 2000).

Finkel et al. (2012) observed that some plants such as *Tamarix* are able to provide an environment highly rich in organic carbon and sufficient moisture to support the growth of microorganisms on leaves even in highly desiccated area such as desert environment. It was also observed that bacteria isolated did not occur in uniform pattern across the leaves sampled, there ought to be a particular interaction and such interaction is said to determine the extent to which human pathogens are able to colonize and survive on plant tissue such as fresh edible produce (Beattie and Marcell, 2002; Whipps et al., 2008), was observed in the pumpkin used in this study. Researchers have also shown that production of Inole-3-acetic acid (IAA), pili, and flagella by phyllosphere bacteria facilitate quick colonization of the leaf surface by increasing the nutrient leakage, microbial fitness and easy attachment to the leaf surface (Brandl et al., 2001; Romantschuck et al., 2002; Lindow and Brandl, 2003). The highest frequency of fungal species isolated from pumpkin phyllosphere was also attributed to the nature of phyllosphere. Pumpkin as said earlier, have broad and less waxy leaves than other plant phyllosphere; this provided a good microhabitat for fungi to grow (Kinkel et al., 2000; Lindow and Brandl, 2003). Members of *Acremonium, Aspergillus, Penicillium* and *Mucor* have been isolated from phyllosphere of some plants (Inacio et al., 2002).

Several studies have shown that in older leaves phyllosphere, filamentous fungi usually occur as spores rather than active mycelia cells (Andrews and Harris, 2000; de Jager et al., 2001). Some plants' leaves are able to produce various volatile organic compounds into the boundary leaf, these compounds which include $CO_2$, acetone, terpenoids, aldehydes, alcohol, hydrocarbons, sesquiterpenoids, sulphides, and nitrogen containing compounds are known to be toxic to fungi and might have prevented and limited their growth on phyllosphere (Mechaber, 2002). These findings supported the results from other studies, which showed that pronounced interspecies variability in phyllosphere communities exit (Yang et al., 2001; Lambais et al., 2006; Whipps et al., 2008). Many studies have also shown that environmental conditions can have important effects on phyllosphere community structure (Lindow and Brandl, 2003). The variation in microbial load from the different sample sites was attributed to natural and human activities in the various sites. Sample site U was more populated and have more trees planted in the sites when compared to the other sample sites used in the study. Activities such as fumigation, antibiosis, antisepsis, disinfection and other medical related activities which prevent pests and microbial growth are usually carried out on routine basis in the sampled sites but sample site U. These activities and several others contributed to the less microbial load from the three hospital sites used in the study.

## Conclusion

The findings from the study showed that phyllospheres are microhabitats, which support the growth of various groups of microorganisms including human pathogens with bacteria being the most abundant among the groups. Most of the edible leafy vegetables which are consumed by human have less waxy phyllosphere, which permit microbial growth; it is necessary that they may be washed and cooked properly before consumption to ensure healthy living.

## Conflict of Interests

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

## ACKNOWLEDGEMENT

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# Genetic evaluation of spring wheat (*Triticum aestivum* L.) genotypes for yield and spot blotch resistance in Eastern Gangetic Plains of India

**Nitish De, D. K. Baranwal* and Sudhir Kumar**

Department of Plant Breeding and Genetics, Bihar Agricultural University (BAU) Sabour Communication No. 006/2014, Sabour Bhagalpur (Bihar) – 813210, India.

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**An experiment was conducted to evaluate 49 spring wheat (*Triticum aestivum* L.) genotypes of diverse origin by estimating genetic parameters viz. variability, character association, path coefficient, cluster and principle component analysis (PCA) for yield and spot blotch disease resistance during 2011 -2012 and 2012 - 2013. Highest phenotypic coefficient of variation (PCV) was observed for area under disease progress curve (AUDPC) (29.15%), plot yield (12.94%) and 1000-kernel weight (11.63%). The highest plot yield (g) was observed in genotypes WH1132 and WH 1131. Grain yield per plot (g) was significantly and positively associated with the 1000-kernel weight (g) (0.82*) and grain per spike (number) (0.79*). Path-coefficient analysis expressed that the maximum positive direct effect on yield showed by grain per spike (number) observed via 1000-kernel weight (g) and days to 75% flowering (days) while negative direct effects showed by 1000-kernel weight (g), AUDPC, days to maturity (days) and plant height (cm). All the 49 spring wheat genotypes were grouped into six distinct clusters. The genotypes of cluster II represented higher yield and disease resistance potential. Out of the major four principal components (PCs), three principal components (PC1, PC2 and PC3) accounted for 79.86% with proportionate values of 45.90, 18.73 and 15.23%, respectively. The third principal component has high positive component value for the days to 75% flowering, the plant height, the AUDPC and the 1000-kernel weight. The breeding objective of the present experiment is to identify genetically diverse wheat genotypes for developing high yielding and disease resistant variety for Eastern Gangetic Plains of India.**

**Key words:** AUDPC, cluster analysis, dendrogram, genetic advance, yield, PCA and PCV.

## INTRODUCTION

Wheat (*Triticum aestivum* L.) is the largest grown and second prominent produced cereal crops worldwide after maize (*Zea mays* L.) with 697.8 million tonnes every year (Anonymous, 2013; Velu and Singh, 2013). Global food production might be increase at least 70% by 2050 when global population may likely to reach 9 billion

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



**Abbreviations: AUDPC,** Area under disease progress curve; **PCA,** principle component analysis.

(Anonymous, 2008). In India, wheat production and productivity was 92.46 million tonnes and 3.12 t/ha, respectively, in 2012 to 2013 which reveal decline trend in comparison with previous year (Anonymous, 2013). Several biotic and abiotic stresses such as spot blotch, leaf rust, terminal heat stress and drought stress have adverse impact on wheat productivity in the eastern regions of South Asia especially Eastern Gangetic Plains of India (Joshi et al., 2007). However, spot blotch is most serious constrain for the wheat production caused by *Cochliobolus sativus* (Ito and Kurib.) Drechsler ex Dastur [anamorph: *Bipolaris sorokiniana* (Sacc.) Shoem results substantial yield loss (Joshi et al., 2007). Present resistance potential against spot blotch disease in high yielded wheat genotypes especially in warmer humid regions of South Asia is still unsatisfactory and need enormous research (Sharma and Duveiller, 2006; Joshi et al., 2007; Meena et al., 2014). Significant negative correlations were observed between spot blotch resistance and some of yield components such as grain yield, 1000-kernel weight, biomass yield, harvest index and grain fill duration (Sharma et al., 1997a).

Under the climate changing scenario, it is a major threat to breeders to sustain food availability for growing population (Kumar et al., 2011). Estimation of heritability and genetic advance is prerequisite for a breeder which helps in understanding the magnitude, nature and interaction of genotype and environmental variation for particular traits. Character association reveals cause of relationship between two variables (Meena et al., 2014). The information obtained by path coefficient analysis helps in indirect selection for genetic improvement of yield because direct selection is not effective for low heritable trait like yield. The cluster analysis and PCA are the basic genetic diversity analysis tools with some relative differences with each other. Primary purpose of cluster analysis is to group individuals based on the characteristics so that individuals with similar characteristics are mathematically collected into the same cluster (Hair et al., 1995; Meena et al., 2014). Determination of optimal and acceptable clusters is another important aspect in cluster analysis. PCA is basically used to sort out the data to establish association between two or more characters by linear transformation of the original variables into a new group of uncorrelated variables regarded as principal components (PCs) (Wiley, 1981). The main objective of present experiment was to acquire information relevant to genetic variability for each trait, heritability, genetic advance, character association, path analysis and genetic diversity. Based on the information, we can propose promising genotypes for further research programmes.

## MATERIALS AND METHODS

The present experiment was conducted at the Bihar Agricultural College (BAC) farm, Sabour, Bhagalpur (Bihar), India during Rabi 2011 to 2012 and 2012 to 2013 utilizing 49 genotypes (Table 1) in simple lattice design with two replications. The experimental site was situated at N 25° 15'and 84° 4' E at the 45.75 m above sea level. Soil pH ranged from 6.5 to 7.5, and the average rainfall in this area is about 1150 mm and average relative humidity is 70% as per meteorological data provided by agro-meteorological observatory, BAC Sabour. Most of the precipitation is usually received during the South-West monsoon season. The 49 spring wheat genotypes were grown under simple lattice design (7 x 7). Gross plot size of each treatment (genotype) was 6.0 m row length and six rows at 23 cm apart (6.0 x 1.38 m). Net plot size of each treatment was 6.0 m row length and four rows excluding border row (6.0 x 0.92 m). Observations were recorded for seven quantitative traits viz. days to 75% flowering (days), days to maturity (days), plant height (cm), 1000-kernel weight. (g), grain per spike (number), area under disease progress curve (AUDPC value) and plot yield (g) through random sampling method. Recommended agronomic package and practices were applied to raise a good crop.

### Area under disease progress curve (AUDPC)

Spot blotch disease was induced by inoculating a pure culture of the locally most aggressive isolate of *B. sorokiniana* following the method of Chaurasia et al. (1999). Spot blotch disease was recorded at different growth stages viz. GS 69 (anthesis complete), GS73 (early milk) and GS 77 (late milk) **(**Zadoks et al., 1974). Disease severity (%) was recorded at different stages to calculate the AUDPC. The AUDPC (Van der Plank, 1963; Roelfs et al., 1992) was calculated using the following formula as:

$$\mathrm{AUDPC} = \sum_{i=1}^{n} \left[ \left\{ \frac{Y_i + Y_{(i+1)}}{2} \right\} (t_{(i+1)} - t_i) \right]$$

Where, Yi is the disease level at time ti and t (i+1) - ti the time (days) between two disease scores and n is the number of dates on which spot blotch was recorded.

### Statistical and biometrical analysis

Data of 10 plants of each treatment were averaged and pooled mean data was used for statistical analysis. Phenotypic and genotypic coefficients of variability were calculated as per method proposed by Burton (1952). Test of significance was estimated using F ratio value at 5% level of statistically significance. Genetic variability including mean, range, variance, CV, heritability (Burton and Devane, 1953) and genetic advance (by Johnson et al., 1955) were calculated. All these analyses were

**Table 1.** Used 49 wheat genotypes with their pedigree and sources.

| Genotype | Pedigree | Source |
|---|---|---|
| Amber 28 | WG5669/2/MACS2496/BOW | KSPL,Jalna (M.H) |
| WCW 2009-06 | PBW 343/WH 147 | SVBPUA&T, Merrut (U.P) |
| HUW 660 | WAXWING*2//INQALAB91*2/KUKUNA | B.H.U, Varanasi (U.P.) |
| NW 5074 | PRL/2 *PASTOR/4/CHOIX/STAR/3/HE1/3*CNO79//2*SERI | NDUA&T., Faizabad(U.P.) |
| DBW 96 | OASIS/SKAUZ//4*BCN/3/2*PASTOR | DWR,Karnal (Haryana) |
| RAJ 4287 | HD3091/Raj3077//Raj3765/UP2338 | SK ,RAU,Jaipur (Rajasthan) |
| UP 2837 | HD 2590/3/PBW 343/UP 1109/UP 2425-10B | GBPUAT, Pantnagar |
| WH 1133 | BABAX/LR 42//BABAX *2/3/VIVITSI | CCSHAU,Hisar (Haryana) |
| PBW 676 | DBW 16/DBW 18 | PAU , Ludhiana |
| PBW 679 | PBW 51/HP 1744 | “ |
| UP 2834 | PBW 503/HPW 89 | “ |
| PBW 343 | ND/VG1944//KAL//BB/3/YACO'S'/4/VEE#5'S' | “ |
| HD 3104 | HD 2329/HDK 10 | IARI, New Delhi |
| HD 3107 | HD 2877/DL 388 | “ |
| RAJ 4285 | Raj4037/HUW570 | SK ,RAU,Jaipur (Rajasthan) |
| WH 1131 | MUNIA/CHTO//AMSEL | CCSHAU,Hisar (Haryana) |
| K 1101 | HD 2733/HD 2285 | CSAUAT, Kanpur,(U.P.) |
| HD 2733 | ATTILA/3/TUI/CARC//CHEN/CHTO/4/ATTILA | IARI, New Delhi |
| DBW 98 | PBW65/2*PASTOR//PBW550 | DWR,Karnal (Haryana) |
| HD 3105 | WAXWING*2VIVITSI | IARI, New Delhi |
| HP 1941 | FRAME//MILAN/KAUZ/3/PASTOR | IARI, R.S., Pusa (Bihar) |
| RAJ 4289 | PBW283/B8//Raj3077/NW2044 | SK ,RAU,Jaipur (Rajasthan) |
| WH 1134 | PRL/2*PASTER | CCSHAU,Hisar (Haryana) |
| HUW 661 | W15.92/4/PASTOR//HXL7573/2*BAU/3/WBLL1 | B.H.U, Varanasi (U.P.) |
| DBW 112 | INQUALAB/30thIBWSN116//HUW593 | DWR,Karnal (Haryana) |
| DBW 17 | CMH79A.95/3*CNO79//RAJ3777 | “ |
| NW 5077 | PFAU/SERI.1B//AMAD/3/WAXWING | NDUA&T, Faizabad(U.P.) |
| RAJ 4286 | W32/Raj3765//B8 | SK ,RAU,Jaipur (Rajasthan) |
| UP 2835 | CROC.1/Ae. 58(205)BORL95/3/2*MILAN/4/KO123 | GBPUAT, Pantnagar |
| WH 1132 | PBW 65/2*PASTER | CCSHAU,Hisar (Haryana) |
| UP 2838 | CHOIXM95/4/NL 962/3/TRACHA-2//CMH.76-252/PVN'S | GBPUAT, Pantnagar |
| PBW 677 | PFAU/MILAN/5/CHEN/A. squa//BCN/3/VEE#7/BOW/4/PAST | PAU , Ludhiana |
| HD 3108 | WHEAR//2*PRL/2*PASTOR | IARI, New Delhi |
| TL 2984 | NGSN23/JNIT141//TL551/M78-9224 | PAU , Ludhiana |
| WH 1135 | HD 29/2*WEAVER | CCSHAU,Hisar (Haryana) |
| DBW 99 | HD2168/HJA70581//HD2590 | DWR,Karnal (Haryana) |
| K 1102 | PBW 343/Raj 3765 | CSAUAT, Kanpur,(U.P.) |
| PBW 680 | PBW 343/Tc+Lr37//PBW 343 | PAU , Ludhiana |
| DBW 95 | K9908/PBW534 | DWR,Karnal (Haryana) |
| HP 1942 | TOBA97/PASTOR | IARI, R.S., Pusa (Bihar) |
| K 307 | K 8321/UP2003 | CSAUAT, Kanpur,(U.P.) |
| JAUW 596 | HD2687/Ae. Crassa//HD2687 | SKAUST,Jammu (J &K) |
| DBW 97 | KAUZ//ALTAR84/AOS/3/MILAN/KAUZ/4/HUITES | DWR,Karnal (Haryana) |
| HD 3106 | PRL/2*PASTER/4/CHOIX/STAR/3/HE.1/3/CNO79//2*SERI | IARI, New Delhi |
| PBW 678 | INQ 91*3/TUKURU//DBW 18 | PAU , Ludhiana |
| UP 2836 | UP 2425/ZANDER 33/PHR 1010 | GBPUAT, Pantnagar |
| RAJ 4288 | Raj4048/Raj3777//Lok1 | SK ,RAU,Jaipur (Rajasthan) |
| HP 1943 | WAXWING*2/VIVITSI | IARI, R.S. Pusa (Bihar) |
| NW 5079 | BABAX/LR 42//BABAX *2/3/PAVON 753+LR 47 | NDUA&T, Faizabad(U.P.) |

**Table 2.** Genetic variability, heritability and genetic advance for examined traits in 49 wheat genotypes.

| Trait | Mean | Range | Vp | Vg | PCV (%) | GCV (%) | $H^2$ (B.S. %) | Genetic advance | GAPM (%) |
|---|---|---|---|---|---|---|---|---|---|
| DF | 76.91 | 68.25 - 85.25 | 26.88 | 10.46 | 6.74 | 4.21 | 38.91 | 4.156 | 5.404 |
| PH | 97.88 | 84.75 - 111.25 | 57.34 | 24.61 | 7.74 | 5.10 | 42.92 | 6.696 | 6.841 |
| TGW | 39.49 | 33.50 - 45.00 | 5.43 | 1.26 | 11.63 | 5.61 | 23.24 | 1.115 | 2.825 |
| GPS | 42.39 | 35.00- 49.00 | 15.59 | 8.81 | 9.32 | 7.00 | 56.47 | 4.594 | 10.837 |
| DM | 123.15 | 118.75 – 126.75 | 5.55 | 1.18 | 1.91 | 0.88 | 21.17 | 1.027 | 0.834 |
| AUDPC | 377.14 | 210 - 620 | 16439.52 | 9355.56 | 29.15 | 21.99 | 56.91 | 150.312 | 39.855 |
| PY | 2067.68 | 1767.50 - 2447.50 | 71611.69 | 22104.96 | 12.94 | 7.19 | 30.87 | 170.163 | 8.230 |

Vp = Phenotypic variance, Vg = genotypic variance, PCV = phenotypic coefficient of variation, GCV = genotypic coefficient of variation, $H^2$ (b.s.) = heritability in broad sense, GAPM = genetic advance in per cent of mean, DF = days to 75% flowering, PH = plant height; TGW = 1000-kernel weight, GPS = grain per spike, DM = days to maturity, area under disease progress curve (AUDPC) and PY = Plot Yield.

al., 1955) were calculated. All these analyses were performed by SPAR 2.0 (Statistical software). Genetic advance as per cent of mean is the improvement in the mean of selected family over the base population (Johnson et al., 1955). Correlation coefficient analysis was calculated by Robinson et al. (1951). The significance of correlation coefficient was tested with the help of 'r' value at n-2 degree of freedom at 5% level of significance where 'n' is number of treatments. Path coefficient analysis was accessed by Dewey and Lu (1959) using SPAR 2.0 (Statistical software). The correlation coefficient analysis and genetic diversity analysis using cluster and PCA were calculated using statistical software of STATISTICA version 10.0.

## RESULTS AND DISCUSSION

In the present experiment, 49 spring wheat (*T. aestivum* L.) genotypes were analyzed for genetic studies viz., the genetic variability, the character association, the cluster analysis and principal component analysis (PCA) for examined yield components (days to 75% flowering, days to maturity, plant height, 1000-kernel weight, grain per spike and plot yield) and spot blotch resistance. It was found that the PCV was slightly higher than the GCV for all studied character revealing the environmental effects on the expression of characters. Highest PCV value was observed for the AUDPC value (29.15%), plot yield (g) (12.94%) and 1000-kernel weight (g) (11.63%) as similar reported by (Ali et al., 2008) (Table 2). It expressed the presence of maximum genetic variability among cultivars. Heritability (B.S.) value was found the highest for character AUDPC (56.91%) followed by grain per spike (grain number) (56.47%) and plant height (cm) (42.92%). Highest heritability value along with maximum genetic advance as per cent of mean was observed for the AUDPC (56.91 and 39.85%) followed by grain per spike (56.47 and 10.83%) and plant height (42.92 and 6.84%) (Table 2), and it indicated the presence of additive genetic effects for expression of these characters; selection considering these characters would be effective. The best 10 genotypes based on mean performance of promising traits in desirable direction, are represented in Table 3. The genotypes TL 2984 and UP 2838 were showed lesser days to 75% flowering (68.25 and 69.75 days). The genotype WH 1134 was exhibited by the lowest AUDPC value (210) indicating resistant parent in consonance with (Sharma et al., 1997b) (Table 3). Low yield level indicates high susceptibility to spot blotch disease (Phadnawis et al., 2002). Highest 1000-kernel weight (g) was observed in HUW 661 and HD 3104 (45.00 g), and the highest grain per spike were found in the TL 2984 (49.00) (Table 3).

Similarly, the highest plot yield was recorded in WH1132 and WH 1131 (Table 3). It shows wide differences among the experimental material in terms of yield components and spot blotch resistant. Grain yield per plot (g) was significantly and positively associated with 1000-kernel weight (g) (0.82*) and grain per spike (0.79*) (Table 4). It suggests that the characters should be included for genetic improvement for spring wheat genotypes.

Negatively significant correlation (-0.689*) was observed between the yield (g) and the AUDPC value indicate that spot blotch is the major constraint for wheat production in Eastern Gangetic Plains of India representing major role of environment for the disease incidence (Table 4) as similar reported by (Gilchrist and Pfeiffer, 1991; Meena et al., 2014) AUDPC value showed negative and significant association with 1000-kernel weight (g) (-0.599*) and grain per spike (grain number) (-0.524*) (Table 4). Path-coefficient analysis exhibited that the maximum positive direct effect on yield showed by grain per spike (grain number) observed via the 1000-kernel weight (g) and days to 75% flowering (days) while negative direct effects showed by the 1000-kernel weight (g), the AUDPC (unit), the days to maturity (days) and the plant height (cm) (Table 5). Thus, path analysis suggest that grain per spike (unit), days to 75% flowering (days), 1000-kernel weight (g), the AUDPC value and the plant

**Table 3**. Best ten genotypes considering promising characters.

| Trait | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| DF **(early)** | **TL 2984 (68.25)** | UP 2838 (69.75) | **WCW2009-06 (70.00)** | HD 3104 (70.50) | HUW 661 (71.75) | RAJ 4285 (72.00) | PBW 678 (72.00) | UP 2836 (72.00) | RAJ 4289 (72.25) | DBW 95 (73.50) |
| PH **(dwarf)** | RAJ 4286 (84.75) | HD 3108 (87.00) | DBW 17 (87.75) | K 1101 (88.75) | NW 5074 (90.25) | UP 2836 (91.00) | PBW 678 (91.50) | DBW 97 (91.75) | NW 5079 (91.75) | PBW 676 (92.00) |
| TGW **(high)** | **HUW 661 (45.00)** | **HD 3104 (45.00)** | DBW 99 (45.00) | PBW 676 (44.00) | WH 1132 (44.00) | **TL 2984 (44.00)** | HP 1943 (43.75) | UP 2836 (43.50) | HD 2733 (43.00) | DBW 112 (43.00) |
| GPS **(high)** | **TL 2984 (49.00)** | HD 2733 (48.00) | PBW 677 (48.00) | WH 1132 (47.50) | PBW 676 (47.00) | **WCW 2009-06 (47.00)** | HUW 661 (46.00) | HD 3106 (46.00) | WH 1131 (46.00) | HD 3104 (45.00) |
| DM **(early)** | HP 1942 (118.75) | **WH 1132 (119.75)** | **WH 1131 (120.75)** | HP 1943 (121.00) | NW 5079 (121.25) | UP 2834 (121.50) | PBW 677 (121.75) | K 1102 (121.75) | WH 1133 (121.75) | DBW 98 (122.00) |
| AUDPC **(low)** | WH 1134 (210.00) | **WCW 2009-06 (240.00)** | DBW 112 (240.00) | K 1101 (250.00) | **WH 1131 (260.00)** | HUW 661 (260.00) | WH 1132 (265.00) | HD 2733 (270.00) | DBW 98 (280.00) | UP 2836 (290.00) |
| PY **(high)** | **WH 1132 (2447.50)** | **WH 1131 (2402.50)** | **HD 3104 (2392.50)** | **HUW 661 (2372.50)** | PBW 676 (2362.50) | **TL 2984 (2360.00)** | **WCW 2009-06 (2330.00)** | HD 2733 (2320.00) | DBW 17 (2260.00) | UP 2836 (2255.00) |

*Bold genotypes are better for several characters. DF: Days to 75% Flowering, PH = plant height; TGW= 1000-kernel weight, GPS = Grain per spike, DM = Days to maturity, Area under disease progress curve (AUDPC value) and PY = Plot Yield.

**Table 4**. Character association among seven characters in 49 wheat genotypes.

| Trait | DF | PH | TGW | GPS | DM | AUDPC | PY |
|---|---|---|---|---|---|---|---|
| DF | 1.000 | 0.041 | -0.019 | -0.133 | 0.253 | 0.053 | -0.057 |
| PH | | 1.000 | 0.180 | 0.105 | -0.321* | 0.077 | 0.081 |
| TGW | | | 1.000 | 0.781* | -0.127 | -0.599* | 0.822* |
| GPS | | | | 1.000 | -0.179 | -0.524* | 0.790* |
| DM | | | | | 1.000 | 0.156 | -0.287* |
| AUDPC | | | | | | 1.000 | -0.689* |
| PY | | | | | | | 1.000 |

*Statistically significant at 5% level. DF = Days to 75% flowering, PH = plant height; TGW = 1000-kernel weight, GPS = grain per spike, DM = days to maturity, AUDPC = area under disease progress curve, PY = plot yield.

height (cm) may serve as effective selection variables for further wheat improvement programmes.

## Genetic divergence analysis

All the 49 spring wheats were grouped into six distinct clusters through STATISTICA V.10 software (Table 6, Figure 1). Based on Euclidean genetic distance, paired entry (DBW96 and WH1132), (DBW96 and WH 1131) and (RAJ4287 and WH1131) were found extremely diverse while paired entry (HUW660 and DBW 98), (PBW679 and RAJ4288) exhibited extremely closest genetic relationship. In cluster I, only one entry Amber 28 found which represent poor yield potential. Seven genotypes are categories under cluster II.

The mean performance of the cluster genotypes for 1000-kernel weight (43.43 g), grain per spike (46.93 grain) and plot yield (2375.0 g) are above the mean of all genotypes and for the AUDPC value below the grand mean representing higher yield and disease resistance potential (Table 7).

Cluster III has 28 genotypes accounting for 57.14% of total genotypes having poor yield and resistance potential in consonance with (Atta et al., 2008; Khan et al., (2010).

**Table 5.** Direct and indirect effect of six characters on plot yield as independent variable.

| Trait | DF | PH | TGW | GPS | DM | AUDPC | Correlation With PY |
|---|---|---|---|---|---|---|---|
| DF | **0.0300** | 0.0008 | -0.0039 | -0.0041 | 0.0063 | -0.0062 | -0.057 |
| PH | -0.0015 | **-0.055** | -0.0093 | -0.0068 | 0.0244 | 0.0032 | 0.081 |
| TGW | 0.0609 | -0.083 | **-0.4901** | -0.5331 | 0.0905 | -0.0099 | **0.822** |
| GPS | -0.2208 | 0.2026 | 1.7583 | **1.6165** | -0.4547 | 0.1098 | **0.790** |
| DM | -0.0192 | 0.0408 | 0.0169 | 0.0257 | **-0.0914** | 0.0374 | **-0.287** |
| AUDPC | 0.0489 | 0.0137 | -0.0048 | -0.0161 | 0.0969 | **-0.2369** | **-0.689** |

Residual effects = 0.415. DF = days to 75% flowering, PH = plant height; TGW = 1000-kernel weight, GPS = grain per spike, DM = days to maturity, AUDPC = area under disease progress curve, PY = plot yield.

**Table 6.** Clustering pattern of genotypes based on dendrogram (cluster analysis tree chart).

| Clusters | No. of genotypes | Genotypes |
|---|---|---|
| Cluster I | 1 | Amber 28 |
| Cluster II | 7 | WCW2009-06, HD2733, HD3104, TL2984, WH1131, HUW661 and WH1132 |
| Cluster III | 28 | HUW660, DBW98, HP1943, K1101, DBW112, UP2837, JAUW596, K1102, PBW680, WH1134, NW5074, UP2835, NW5077, DBW 97, PBW679, NW5079, RAJ4288, K307, DBW95, HD3106, HP1941, RAJ4286, RAJ 4289, RAJ4285, UP2838, HD3108, WH1135 and PBW678, |
| Cluster IV | 8 | WH1133, HP1942, DBW99, HD3107, HD3105, PBW677, DBW17 and UP2836 |
| Cluster V | 2 | DBW96 and RAJ4287 |
| Cluster VI | 3 | PBW676, UP2834 and PBW343 |

The cluster IV has eight genotypes accounting for 16.32% of total genotypes. The mean performance of the cluster genotypes for the plant height (cm), 1000-kernel weight (g), grain–per spike (grain number) and plot yield (g) was higher than grand mean value and for the AUDPC value lower than the grand mean value representing better yield and resistant potential.

The cluster V has two genotypes representing poor yield potential while the cluster VI with three genotypes exhibited better yield and resistant potential because mean performance of the cluster genotypes for the plant height (cm), the 1000-kernel weight (g), the grain per spike (grain number) and the plot yield (g) was higher than grand mean value and for the AUDPC lower than the mean value (Table 7).

## Principal components analysis (PCA)

It is generally used for data reduction to ascertain the relationship between two or more characters by linear transformation of the original variables into a new group of uncorrelated variables regarded as PCs. Four major PCs (PC1 to PC4) from the original data explained 89.13% of the total variation (Table 8) as similar reported by Hailegiorgis et al. (2011) and Meena et al. (2014). Out of the major four PCs, three principal components (PC1, PC2 and PC3) accounted with proportionate values of 45.90, 18.73 and 15.23%, respectively and contributed 79.86% of the cumulative variation having Eigen value more than one (Table 8). Two dimensional depictions of 49 wheat genotypes on PC axis 1 and 2 represented the existence of extreme genetic diversity among present wheat genotypes set (Figure 2).

The first principal component has high positive component value for AUDPC value, days to 75% flowering (days) and days to maturity (days). PC1 has negative component value for 1000-kernel weight (g), grain per spike (grain number) and plot yield (g) as similar reported by Khodadadi et al. (2011) and Meena et al. (2014).

The second PC has high positive component value for days to maturity (days), days to 75% flowering (days) and 1000-kernel weight (g) and high negative component value for plant height and AUDPC (Table 8). These characters having either high positive or negative component value reveals tremendous genetic diversity, and might be play significant role during clustering. The third PC has high positive component value for days to 75% flowering (days), plant height (cm), AUDPC value and 1000-kernel weight (g) (Table 8) as similar reported by Hailegiorgis et al. (2011).

The depiction of component traits on PC1 and PC2 represented that 1000-kernel weight and grain per spike (grain number) are positively related with grain yield (unit) and negative relation exhibited by the AUDPC value (Figure 3).

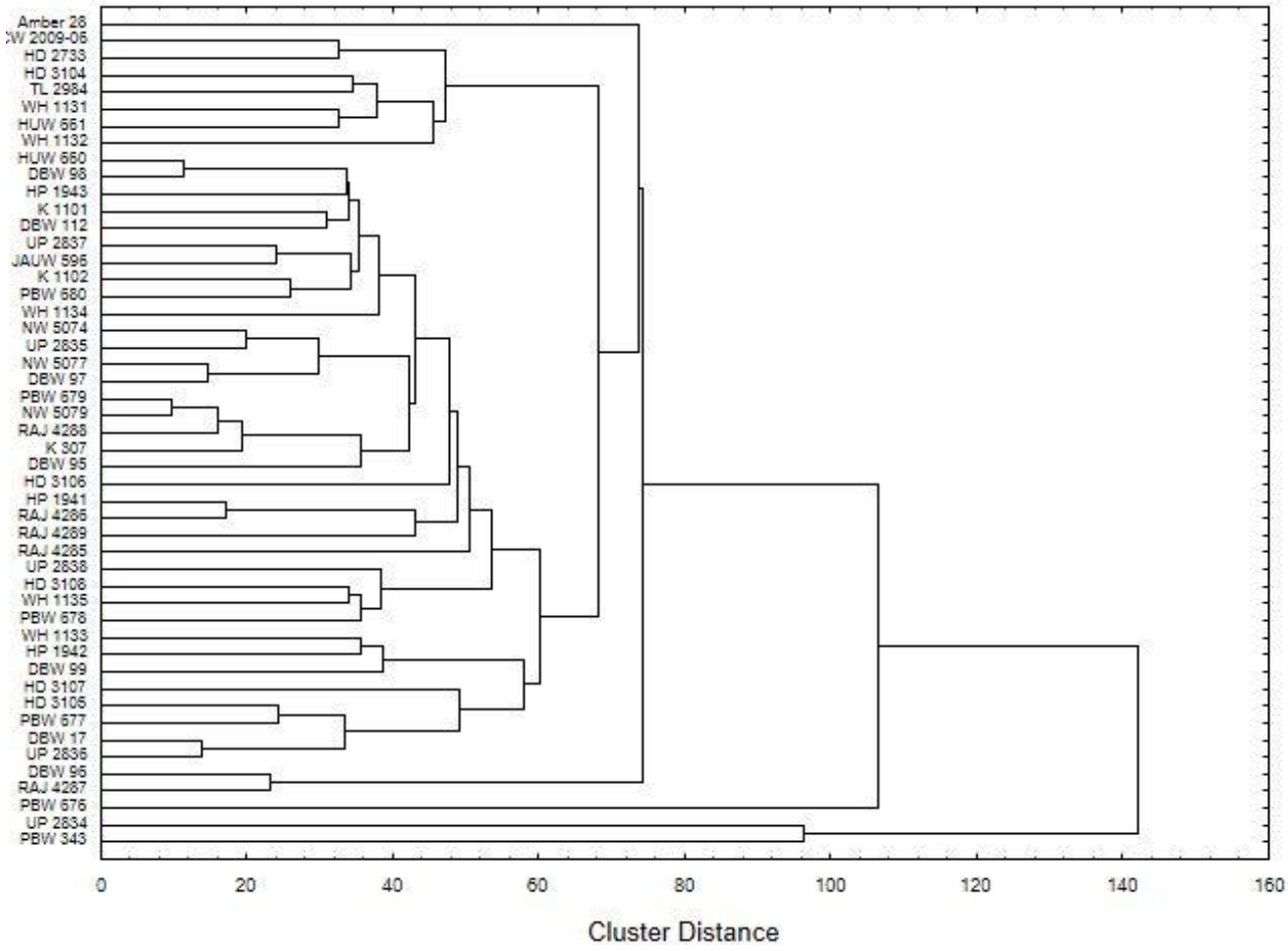


**Figure 1.** Dendrogram depicting genetic diversity among 49 wheat genotypes.

**Table 7.** The mean performance of each cluster parent (above number) and its deviation from grand mean (below number).

| | DF | PH | TGW | GPS | DM | AUDPC | PY |
|---|---|---|---|---|---|---|---|
| Cluster1 | 74.50 | 97.75 | 33.50 | 42.00 | 123.25 | 570.00 | 1848.75 |
| | -2.41 | -0.13 | -5.98 | -0.39 | 0.10 | 192.86 | -218.93 |
| Cluster2 | 73.29 | 97.61 | 43.43 | 46.93 | 122.21 | 270.71 | 2375.00 |
| | -3.62 | -0.28 | 3.94 | 4.54 | -0.94 | -106.43 | 307.32 |
| Cluster3 | 76.90 | 97.04 | 38.19 | 40.95 | 123.34 | 376.79 | 1971.34 |
| | -0.01 | -0.84 | -1.30 | -1.44 | 0.19 | -0.36 | -96.34 |
| Cluster4 | 79.64 | 100.64 | 41.43 | 43.86 | 122.54 | 340.71 | 2203.21 |
| | 2.73 | 2.76 | 1.94 | 1.47 | -0.62 | -36.43 | 135.54 |
| Cluster5 | 80.50 | 99.13 | 35.50 | 38.50 | 125.88 | 600.00 | 1772.50 |
| | 3.59 | 1.24 | -3.98 | -3.89 | 2.72 | 222.86 | -295.18 |
| Cluster6 | 79.08 | 101.42 | 41.17 | 43.67 | 123.00 | 530.00 | 2140.83 |
| | 2.18 | 3.53 | 1.68 | 1.28 | -0.15 | 152.86 | 73.15 |

**Table 8.** Principal components analysis (PCA) for seven examined characters in 49 wheat genotypes.

| Trait | PC1 | PC2 | PC3 | PC4 |
|---|---|---|---|---|
| Eigen value | 3.213 | 1.311 | 1.066 | 0.649 |
| Cumulative | 3.213 | 4.524 | 5.590 | 6.239 |
| % Total variation | 45.903 | 18.730 | 15.230 | 9.270 |
| Cumulative | 45.903 | 64.633 | 79.862 | 89.133 |
| Days to 75% flowering | 0.077 | 0.382 | 0.778 | 0.438 |
| Plant height | -0.095 | -0.587 | 0.591 | -0.344 |
| 1000-kernel weight | -0.502 | 0.108 | 0.143 | -0.237 |
| Grain per spike | -0.490 | 0.060 | -0.008 | -0.292 |
| Days to maturity | 0.189 | 0.666 | 0.091 | -0.623 |
| AUDPC value | 0.427 | -0.211 | 0.128 | -0.392 |
| Plot Yield | -0.524 | 0.071 | 0.015 | 0.082 |

DF = Days to 75% flowering, PH = plant height; TGW = 1000-kernel weight, GPS = grain per spike, DM = days to maturity, AUDPC = area under disease progress curve, PY = plot yield.

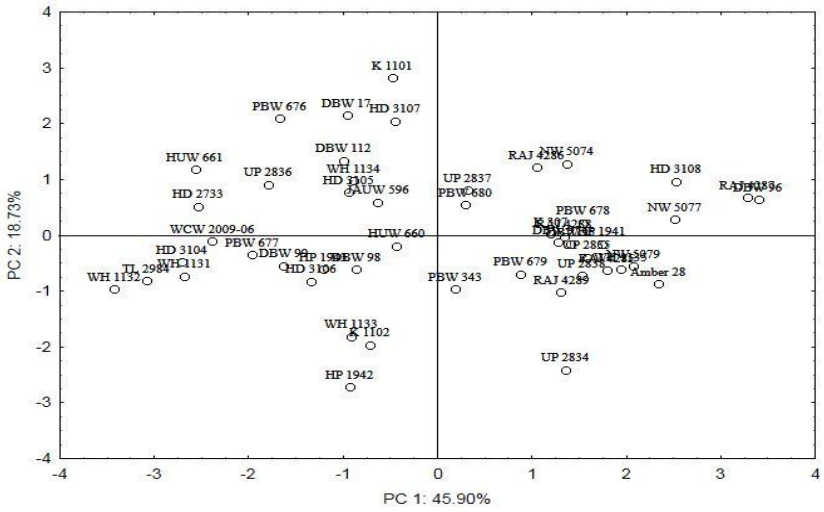


**Figure 2.** Scatter plot: Two dimensional depictions of 49 wheat genotypes on PC1 and PC2 axis.

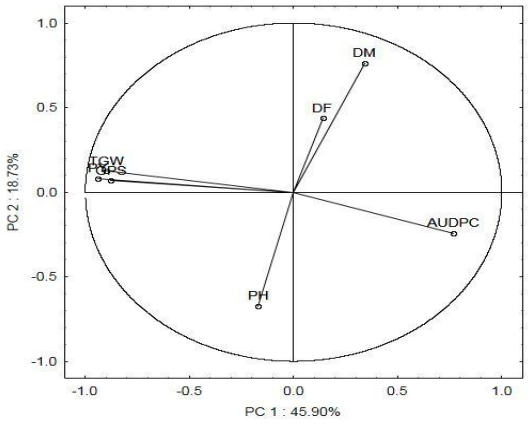


**Figure 3.** Scattered diagram based on first two principal components representing contribution of the examined characters. DF = Days to 75% flowering, PH = plant height; TGW= 1000-kernel weight, GPS = grain per spike, DM = days to maturity, AUDPC = area under disease progress curve and PY = Plot Yield.

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# Certain growth related attributes of bunchy top virus infected banana under *ex-vitro* conditions

**Ikram-ul-Haq[1]*, Shahrukh[1], Um-E-Aiman[1], Saifullah Khan[2], Nazia Parveen[3], Madiha Fatima[1] and Muhammad Umar Dahot[1]**

[1]Institute of Biotechnology and Genetic Engineering (IBGE), University of Sindh, Jamshoro-76080, Pakistan.
[2]Plant Tissue Culture and Biotechnology Wing, H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan.
[3]Department of Statistics, University of Sindh, Jamshoro-76080, Pakistan.

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**Effect of banana bunchy top virus (BBTV) on morpho-physiological characteristics of banana (*Musa* sp.) cv., Basrai plants was assessed. Healthy and BBTV infected samples of banana were collected from its open fields and micro-propagated aseptically. These plantlets were established in wire-house for three months. Presence of BBTV was confirmed by polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). Micro-propagation efficiency of BBTV-infected plants was observed in less than healthy plants under *in-vitro* as well as *in-vivo*. Significant reduction in plant height, fresh weight (F wt) and dry weight (D wt) of leaf was observed in BBTV infected plants. A co-relation of peroxidase (POX) activity was observed with total carotenoids that increased in BBTV infected plants, while chlorophyll contents decreased significantly. Nitrate reductase activity also decreased with increase in proline contents in BBTV-stresses plants ($p<0.05$). Meanwhile, reducing sugars also increased but not-significantly. Bunchy top virus infection in banana therefore resulted in alteration of growth related physiological traits that led to retardation of plant growth.**

**Key words:** *Ex-vitro*, *Musa* sp., micro-propagation, photosynthetic pigments, BBTV, peroxidases, total proteins, reducing sugars, cell size.

## INTRODUCTION

In plants, a number of environmental stresses (biotic and abiotic) produce characteristically identifiable symptoms because of deleterious impacts on different physiological processes. Each stress has developed a number of bio-chemical changes (Miteva et al., 2005). Viral pathogens (biotic stress) have also caused severe damage in many

*Corresponding author. E-mail: rao.ikram@yahoo.com. Tel: +92-345-2914291.



**Abbreviations: MS,** Murashige and Skoog; **BAP,** benzyleaminopurine; **PCR,** polymerase chain reaction; **ELISA**, enzyme-linked immunosorbent assay; **BBTV,** banana bunchy top virus; **RWC,** relative water contents; **F Wt,** fresh weight; **D Wt,** dry weight; **NRA,** nitrate reductase activity; **POX,** peroxidases; **$Na_2CO_3$,** sodium carbonate.

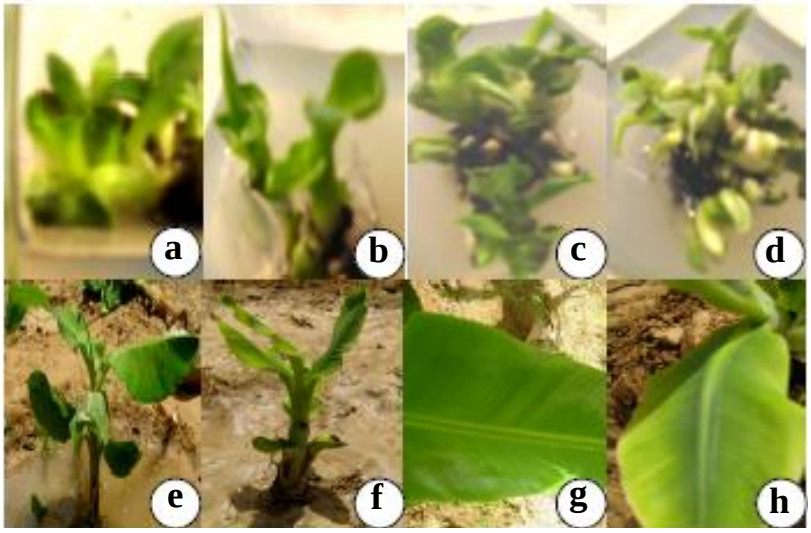


**Figure 1.** *In-vitro* and *ex-vitro* multiplying BBTV-infected and healthy banana (*Musa* sp.) cv., Basrai plants under experimental conditions. a and b, 1st micro-propagation culture of the non-infected and BBTV infected suckers; c and d, 3rd sub-cultures respectively; e and f, six weeks old healthy and infected banana plants growing in wire-house, respectively; g and h, 3rd leaf from top to bottom of three-months old healthy and infected plants, respectively. BBTV, Banana bunchy top virus.

major crops either by reducing yields or quality of plants as a whole (Kang et al., 2005). Presently, banana is affected by five growth limiting viruses (Stover, 1972), with the most destructive being the banana bunchy top virus (BBTV) transmitted through planting infected banana nursery and by banana aphids (*Pentalonia nigronervosa*) to healthy plants. This virus is observed around the world, especially in Asia and the Pacific regions (Magee, 1940; Wu, 1978; Dale, 1987; Su, 1990; Harding et al., 1991; Moffat, 2001). Symptoms of this viral infection become visible when virus severity in infected tissues reaches to critical point. The BBTV infected plants have been noted with stunted growth, deformation of young leaves and bunched at top of plant (Chia et al., 1992, 1995; Hendry, 1987).

Virus-free plants show relatively better vegetative growth in field, but reduce with viral infection due to decreased photosynthetic surface, as well as respiration rate (Balachandran et al., 1997; Guo et al., 2005). In general, the plant growth of virus infected plants is limited because of inhibitions of biological system in communication to growth related metabolic processes. Similarly, POX enzymes play an integral role in plant metabolism and immune system from seed germination to maturity and become highly active during senescence (Siegel, 1993). The plants immune system is primarily dependent on POX and show hypersensitivity response. Systemic viral infection may lead to increase in defense activity, as well as wound repair by POX (Ye et al., 1990; Candela et al., 1994). Meanwhile, necrosis or chlorosis appears by virus infection (Wood, 1990). In higher plants, both biotic and abiotic stresses have produced a number of characteristic changes in plant morphs and metabolic processes. For example, tissues' POX activity increases because of toxic elements present in cells (Espelie et al., 1986; Stroinski, 1995; Miteva et al., 2005).

In the present experiment, morpho-metabolic attributes were quantified in BBTV infected and virus-free banana plants (*Musa* sp.) comparatively. BBTV infection developed a number of specific abnormal traits in multiplied banana plants under *in-vitro* and *ex-vitro* also. Identification of these characters may be helpful to the studies of future amelioration for BBTV resistance in banana crop.

## MATERIALS AND METHODS

The BBTV infected and healthy suckers of banana (*Musa* sp.) cv., Basrai were collected from different open field banana farms. Inner meristematic regions were excised from suckers and used as explants for micro-propagation (Haq and Dahot, 2007a, b). Micro-propagated plantlets were propagated for three sub-cultures and rooted. These plantlets were screened for presence of BBTV through enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) (Figure 2) (Ennos, 2000; Haq et al., 2009) and shifted to wire-house for further development. When plants were of three months old, they were subjected to different morpho-physiological studies (Figure 1). The location of this experiment was far from the nearest banana field about 60 km. *Ex-vitro* propagating plants were sprayed twice (one-month interval) with imidacloprid (Provado®) at 60 ppm to control banana aphids by using small farmer's hand-pump (Robson and Wright, 2007).

Number of leaves was counted and plant height was taken from the sucker to petiole of fully emerged uppermost leaf and pseudo-

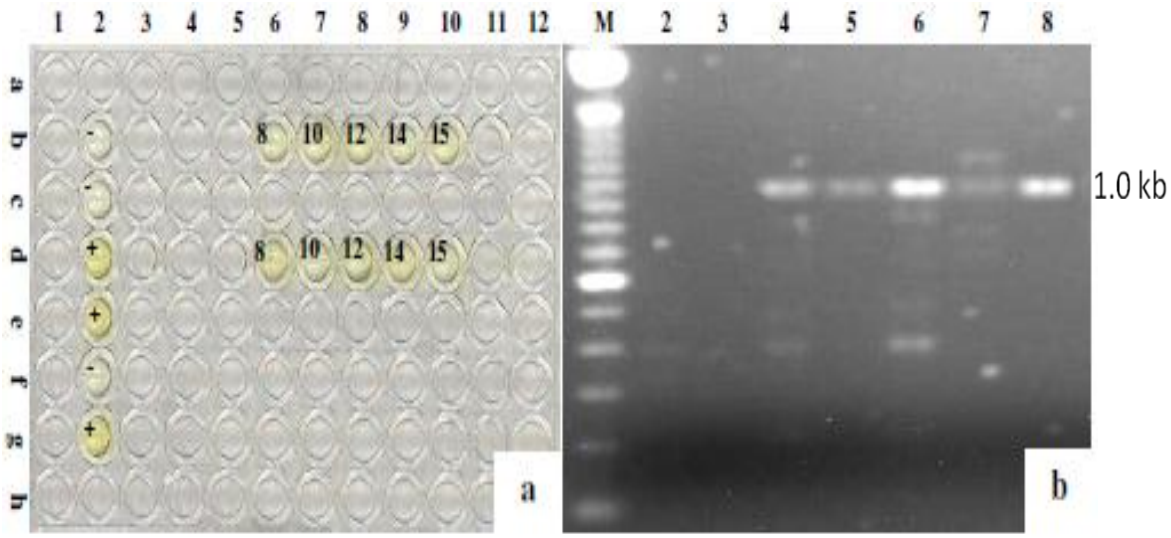


**Figure 2.** Detection of BBTV in aseptically micro-propagated banana (*Musa* sp.) cv. Basrai plantlets before establishing in wire-house. a, ELISA of five-BBTV infected plants in comparison to controls [positive (+ve) and negative (–ve)]; b, PCR based detection of BBTV infection in same 5 BBTV infected plants in comparison to controls (+ve and –ve); lane M, 100 bp DNA marker; lane 2, water; lane 3, -ve plant control; lanes 4 to 8, represent the five samples (8, 10, 12, 14 and 15) respectively. BBTV, Banana bunchy top virus.

stem diameter at centered point of plant height (Smith et al., 2000). Petiole related parameters were quantified from base of leaf blade to its attachment-point with pseudostem (Ennos, 2000). Leaf area (3$^{rd}$ leaf from top) was measured by multiplying, maximum width of leaf with length and conversion factor (0.83). Fresh weight (F Wt) and dry weight (D Wt) of 100 leaf disks (6.3 mm) were taken and relative water contents (RWC) also calculated (Robinson and Neil, 1985; Conroy et al., 1988). Moreover, the third leaf from top of developing banana plants was used for different bio-chemical and anatomical studies. It was chopped into small fragments (2 to 3 mm in length) and fixed in fixation buffer [3.5% glutaraldehyde, 0.1 M phosphate buffer (pH 7.0)] for 10 h at room temperature. Mixture was washed with 0.1 M phosphate buffer twice and again fixed in osmium tetra-oxide (2.5%) overnight (Gielwanowska et al., 2005). Free hand sections were cut from the material for further analysis (Johansen, 1940).

In addition, chlorophyll contents were determined by using Arnon's method (1949), while for determination of POX activity, 1.0 g plant material was homogenized with 3.0 ml sodium phosphate buffer (66 mM; pH 6.1) according to the method of Ranieri et al. (1995) and analyzed as by Curtis (1971) and Bergmeyer et al. (1974). The nitrate reductase activity (NRA) was determined as 100 mg young leaf tissue in phosphate buffer. A reaction mixture was raised by mixing 1.0 ml of 0.1 mM potassium phosphate, 0.5 ml of 0.05 M potassium nitrate ($KNO_3$) and 1% isopropanol (v/v) with pH 7.5 at 30°C in dark. After 1 h, 1.0 ml sulfanilamide (1%) and 1.0 ml naphthyl ethylenediamine dihydrochloride (0.02%) were also added. The nitrate contents were quantified at 540 nm (Klepper et al., 1971).

More also, proline (Bates et al., 1973), total carbohydrates (Dubois et al., 1956; Ciha and Brun, 1978), reducing sugar (Miller, 1959), protein (Bradford, 1976) and nitrate (Morris and Riley, 1963) were determined. For phenol determination, 0.1 g young leaf tissues were homogenized in 25 ml commercial ethanol (95%) according to Ozyigit et al. (2007) and was kept at 0°C for 48 h. Absorbance was taken after making its complex by adding 0.5ml Folin-Ciocalteu (50%) and 1.0 ml sodium carbonate [$Na_2CO_3$ (5%)] at 760 nm against 95% ethanol. Furthermore, the cations [sodium ion ($Na^+$), potassium ion ($K^+$) and calcium ion ($Ca^{2+}$)] were determined in leaf midrib by acidic digestion (Wolf, 1982; Malavolta et al., 1989), while chloride contents was measured following the manual of chlorometer.

#### Statistical analysis

Collected data was computed for significant measurements using COSTAT computer package (CoHort Software, Berkeley, USA) at 5% level. Each of the two types of banana plants, healthy (control) and BBTV (infected), had at least seven replicates.

## RESULTS AND DISCUSSION

During this experiment it was observed that BBTV had differential effects on plant growth of banana from early stages to its maturity (Figure 1). Severity of symptoms increased with the increase in age of plant due to the decreased growth potential of developing plants under viral stress. Of course, leaf morphology was affected significantly. Petiole distance decreased ($p<0.05$), while its width and length also decreased but non-significantly. With the decrease in canopy size (non-significantly), leaf production rate was also low in the BBTV infected plants. Meanwhile, size of the structural cells also decreased (non-significantly), while leaf area decreased in BBTV infected plants significantly (Figure 3 and Table 1). Collectively, all of these characters resulted in the decrease in plant height of the BBTV infected plants (Ennos et al., 2000; Kang et al., 2006).

Imbalance ionic toxicity causes abnormalities in combination with all metabolic functions of plants. A

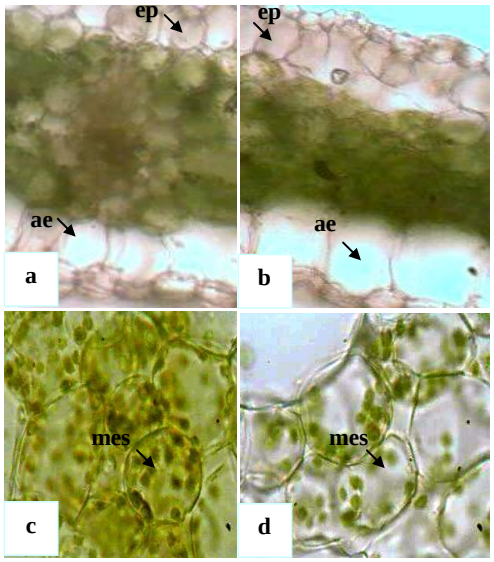


**Figure 3.** Different structural cells, ep, epidermal; mes, mesophyll; ae, aerenchyma; from three- months old BBTV infected plants of banana (*Musa* sp.) cv., Basrai. a and b, The structural cells of healthy and infected banana leaves; c and d, mesophyll cells of healthy and infected banana leaves respectively.

**Table 1.** Certain comparative morpho-physio-anatomical attributes of three-months old BBTV infected banana (*Musa* sp.) cv., Basrai plants.

| Parameter/character | | Control | BBTV Infected |
|---|---|---|---|
| **A. Morphological parameter** | | | |
| 1 | No. of plantlets/plant | 3.95 ± 0.075 | 1.38 ± 0.144** |
| 2 | Plant height (cm) | 69.52 ± 2.65 | 51.49 ± 1.63** |
| 3 | 3rd leaf area ($cm^2$) | 1513.17 ± 14.9 | 1267.24 ± 27.89** |
| 4 | Fresh weight (g) | 1.892 ± 0.06 | 1.561 ± 0.04** |
| 5 | Dry weight (g) | 0.538 ± 0.03 | 0.469 ± 0.03$^{ns}$ |
| 6 | Pseudostem diameter (cm) | 8.24 ± 0.275 | 7.95 ± 0.268$^{ns}$ |
| 7 | Canopy size (cm) | 140.21 ± 4.39 | 129.25 ± 4.18$^{ns}$ |
| 8 | Leaf production rate | 1.42 ± 0.05 | 1.35 ± 0.05$^{ns}$ |
| **B. Leaf petiole measurements** | | | |
| I | Petiole length (cm) | 105 ± 5.60 | 90.21 ± 2.79$^{ns}$ |
| II | Petiole distance (cm) | 10.12 ± 0.12 | 8.59 ± 0.06*** |
| III | Petiole width (cm) | 5.24 ± 0.12 | 4.89 ± 0.27$^{ns}$ |
| **C. Chlorophyll contents (mg $g^{-1}$ F Wt)** | | | |
| a | Chlorophyll a | 0.123 ± 0.006 | 0.066 ± 0.001* |
| b | Chlorophyll b | 0.112 ± 0.002 | 0.072 ± 0.002** |
| c | Chlorophyll ab | 0.235 ± 0.015 | 0.138 ± 0.013** |
| d | Total carotenoids | 1.625 ± 0.013 | 1.72 ± 0.006* |
| **D. Cell size (µM)** | | | |
| i | Epidemal cells | 0.257 ± 0.008 | 0.245 ± 0.002$^{ns}$ |
| ii | Mesophyll cells | 1.612 ± 0.007 | 1.45 ± 0.113$^{ns}$ |
| iii | Aerenchyme calls | 3.252 ± 0.005 | 2.50 ± 0.34$^{ns}$ |

$^{ns}$Non-significant; *,**,***statistically significant. BBTV, Banana bunchy top virus; F Wt, fresh weight

**Table 2.** Some enzymic and bio-chemical characters in three- months old BBTV infected banana (*Musa* sp.) cv., Basrai plants.

| Parameter/character | | Control | BBTV Infected |
|---|---|---|---|
| **A. Organics (mg $g^{-1}$)** | | | |
| a | Protein contents (mg $g^{-1}$) | 0.423 ± 0.011 | 0.537 ± 0.006** |
| b | Reducing sugars (mg $g^{-1}$) | 0.045 ± 0.005 | 0.063 ± 0.003$^{ns}$ |
| c | Total sugars (mg $g^{-1}$) | 0.135 ± 0.002 | 0.152 ± 0.004* |
| d | Proline (mg $g^{-1}$) | 0.245 ± 0.003 | 0.272 ± 0.01* |
| e | Phenol ( mM $g^{-1}$) | 0.584 ± 0.035 | 0.602 ± 0.069$^{ns}$ |
| f | ELISA complex (mg $g^{-1}$) | 0.0 | 0.365 |
| g | RWC (%) | 55.72 ± 2.85 | 53.79 ± 2.71$^{ns}$ |
| **B. Enzymes activity** | | | |
| 1. | **POX activity** (U $min^{-1}$ mg $protein^{-1}$) | | |
| | a. Soluble | 4.67 ± 0.02 | 7.98 ± 0.38* |
| | b. Ionicaly-bounded | 2.25 ± 0.76 | 6.25 ± 0.41** |
| | c. Covalently bounded | 6.93 ± 0.46 | 11.78 ± 0.51** |
| 2. | NRA (mg $NO_2$ $g^{-1}$) | 0.962 ± 0.01 | 0.841 ± 0.05*** |
| **C. Inorganics (mg $g^{-1}$ D Wt)** | | | |
| i | $Na^+$ | 2.92 ± 0.05 | 3.03 ± 0.321$^{ns}$ |
| ii | $K^+$ | 7.39 ± 0.04 | 6.658 ± 0.348$^{ns}$ |
| iii | $Ca^{2+}$ | 6.11 ± 0.059 | 5.356 ± 0.325$^{ns}$ |
| iv | $Cl^-$ | 3.25 ± 0.035 | 3.447 ± 0.134$^{ns}$ |
| v | $NO_3^-$ | 0.105 ± 0.003 | 0.094 ± 0.006$^{ns}$ |

$^{ns}$Non-significant; *,**,***statistically significant. BBTV, Banana bunchy top virus; RWC, relative water contents; D Wt, dry weight; NRA, nitrate reductase activity; $Na^+$, sodium ion; $K^+$, potassium ion; $Ca^{2+}$, calcium ion; D Wt, dry weight; POX, peroxidase.

decreased order of $K^+$, $Ca^{2+}$ and nitrate ($NO_3^-$) was observed to be interlinked with $Na^+$ and $Cl^-$ inverse proportionally (Table 2). Decrease in relative water contents (RWC) was also observed (Table 2). Deficiency in water contents causes a decrease in leaf turgor or hydraulic pressure on which various physiological processes and morphological traits depend like as stomatal opening and growth of leaves (Tecsi et al., 1996; Balachandran et al., 1997). Furthermore, the BBTV infection in banana caused a decrease in green pigments such as chlorophyll (Chl) a, b and ab. Systemic chlorosis due to BBTV infection causes a decrease in rate of photosynthetic processes in leaves. The Chl a have been considered as more sensitive than Chl b in BBTV infected plants (Table 1). The systemic infection caused by plant viruses may be acting as inhibitors for certain enzymes involved in biosynthesis of chlorophyll contents (Goodwin and Britton, 1988; Tecsi et al., 1996). BBTV continuously spread within vascular system and reduces plant growth or accelerate senescence. Moreover, total carotenoid contents increased significantly, which was a typical sign of senescence (Sutic and Sinclair, 1990; Valjakka et al., 1999).

In addition, accumulation of reducing sugars in infected plants was higher than healthy plants as had been widely assumed to be a response of pathogen stress (Table 2). Similarly, significant increase in proline contents in the BBTV infected plants is also a well known indicator of environmental stress (Table 2). Increased proline content may ameliorate the impact of certain stresses (Dorffling et al., 1990; Csonka and Hanson, 1991). Generally, it has been considered that accumulation of proline content is a typical plant osmotic stress response marker, as well as against biotic stress (Table 2). Increase in free proline and protein contents in leaves plant have been observed to be activated naturally under specific hypersensitive response against microbes, including viruses (Goodman et al., 1986; Shalitin and Wolf, 2000). A relationship between reducing sugars, proline contents and total carotenoids was observed as each was increased in infected plants than healthy plants. Phenolic contents in infected plants were also higher than control plants (Table 2).

Moreover, plants are able to tolerate certain environmental stresses to an extent because of accumulation of

either inorganic or low molecular weight organic compounds. Their relative contribution varies from plant to plant, but they play a crucial rule in the plants growing under various stresses (Ashraf and Harris, 2004). For example, POX in healthy plants were observed to remain inactive (covalent-bonding forms), but active (soluble and ionic-bonding forms) in BBTV-infected plants (Table 2). The POX activity was higher in infected plants, while nitrate reductase activity was low in BBTV infected plants. POX therefore remained active and developed a relationship with the chlorophyll contents. It is observed that when chlorophyll content decreases, POX activity increases in virus infected plants but proportioned to total carotenoids (Tables 1 and 2). These alterations are involved in developing pathogen resistance mechanisms (Kuroda et al., 1990; Wood, 1990; Chittoor et al., 1999; Milavec et al., 2001; Wang et al., 2010).

According to Kuroda et al. (1990), POX is responsible for chlorophyll degradation during senescence. Similarly, Smart (1994) reported that chlorophyll degradation occurs due to increase in hydrogen peroxide ($H_2O_2$) and phenolic contents in leaves (Table 2). The horseradish POX is responsible for the catalyses of magnesium ion ($Mg^{2+}$) removal from the precursor of chlorophyll a and chlorophyllin (Azuma et al., 1999). Still, the breakdown of chlorophyll contents through POX pathways in the senescent leaves is dubious (Matile and Hörtensteiner, 1999). In senescent leaves, a variant POX activity has been observed in different plant species during plant growth. Johnson-Flanagan and Spencer (1996) also reported that at maturity stage, plant de-greening occurs. At that time the degradation of the green pigments was associated with increased activity of POX, while low chlorophyllase activity in case of canola has been measured. However, POX and an oxidase in thylakoids of barley leaf are activated for degradation of chlorophyll contents during senescence (Kuroda et al., 1990).

## Conclusion

In this experiment, plant growth decreased in BBTV infected plants due to reduction in synthesis of photosynthetic pigments. Rate of photosynthesis was associated directly or indirectly with a series of metabolic activities. Systematic viral infection caused inhibition of photosynthetic related processes. Meanwhile, significant increase in total carotenoids occurred with the increase in severity of BBTV infection. Accumulation of carotenoids and activation of POX are dependent on the inhibition of different physiological processes due to viral infection. Such loss of biological systems therefore triggers the visibility of specific coloring and become first symptom of BBTV infection than loss of yield and death of plants.

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# Comparative evaluation of organic and conventional farming on chemical quality parameters and antioxidant activity in fruits

**Danielle dos Santos Bonfim de Castro*, Lana de Souza Rosa, Ellen Mayra da Silva Menezes and Anderson Junger Teodoro**

Nutritional Biochemistry Laboratory Core, Food and Nutrition Master Program, UNIRIO, Rio de Janeiro, Brazil.

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**The aim of this research was to determine some important quality parameters and the antioxidant activity of organic and conventional oranges, limes and apples. Five samples of organic fruits with seal certification, organic fruits without seal certification and conventional fruits were acquired from supermarkets and farm in Rio de Janeiro, Brazil. Organic lime and orange showed higher mean values of acidity, being 4.5 and 34.8% higher, when compared to conventional fruit, respectively. There were no statistical differences ($p>0.05$) among organic lime and orange with seal certification, organic without seal certification and conventional fruits regarding the values of density and ascorbic acid. Total soluble solids of organic with seal certification and organic without seal certification in lime was 47.4 and 25.8% higher than conventional fruits. Few statistical differences were observed in acidity and total sugar for Sweet orange (*Citrus sinensis* L. Osbeck) and Lime (Citrus aurantifolia (Christm) Swingle). In relation to the apple samples, there is no significant difference between the physico-chemical analyzes of organic and conventional cultivars. Regarding antioxidant activity, an increase in the percentage of reduction of DPPH (1,1-diphenyl-2-picrylhydrazyl) radical was observed in organic with seal certification lime (18.4%) and orange (22.2%) in comparison to conventional fruit. In general, small differences were observed in chemical quality parameter contents of organically and conventionally grown fruits, however, organic fruits, sealed or not, showed higher antioxidant activity than conventional fruits, suggesting that modulation of these parameters due to alterations in the cultivation practice is unique to the specific species and cultivar of vegetables.**

**Key words:** Organic, orange, lime, apple, antioxidant activity, quality parameters.

## INTRODUCTION

The development of chronic diseases, such as cardiovascular diseases (CVD), cancer, hypertension and type 2 diabetes involves large production of free radicals leading to oxidative stress. A diet rich in fruits and vege-

*Corresponding author. E-mail: danielle.bonfim@hotmail.com. Tel: +55 21 25427276. Fax: +55 21 25427752.

tables has been associated with lower risk of chronic diseases, because in addition to its vitamin and mineral composition, it may also contain other com-pounds with protective effects, in particular antioxidants (Arts and Hollman, 2005).

Polyphenol quantity and quality in plant foods can vary significantly according to different intrinsic and extrinsic factors such as plant genetics and cultivar, soil composition and growing conditions, maturity state and post harvest conditions (Jaffery et al., 2003). The high antioxidant capacity makes polyphenols an important part of a plant's innate defense mechanism, with its synthesis stimulated under stress conditions, such as temperature alterations, UV exposure and pathogenic attacks (Dixon and Paiva, 1995).

Synthetic pesticides and fertilizers are used in the traditional fruit production, whereas the organic agriculture, in general, is characterized by the absence of these products throughout the cultivation period. The literature suggests that organic agriculture could result in foods with higher polyphenol quantity, mainly for two reasons. First, the use of synthetic fertilizers could offer more bioavailable sources of nitrogen, accelerating plant development and plant resources from production of secondary metabolites to growth. Second, the absence of synthetic pesticides could result in higher exposure of the plant to stressful situations leading to an enhancement of natural defense substances such as phenolic compounds (Winter and Davis, 2006; Woese et al., 1997). Both hypotheses would result in foods with higher antioxidant capacity as a consequence of the higher polyphenol composition. The literature showed mixed results regarding the phytochemical composition and antioxidant capacity of organic and conventional vegetables varying according to the bioactive compounds measured and food type (Carbonaro et al., 2002; Lombardi-Boccia et al., 2004).

The debate about the quality parameters superiority of organically and conventionally grown products has been gathering momentum for some time especially in developed countries. The debate has cut across different agricultural produce, animal products inclusive. Organic products are those products which are produced under controlled cultivation conditions with the provisions of the regulation on organic farming. Requirements for organic certification vary from country to country and generally involve a set of production standards for growing, storage, processing, packaging and shipping. There are only few well-controlled studies that are capable of making valid comparison and therefore, compilation of results is difficult and generalization of the conclusions should be made with caution (Masamba and Nguyen, 2008).

The International Federation of Organic Agriculture Movements (IFOAM) fully implemented their standards for organic crop production outlining the criteria that must be met to label agricultural products as "organic" (IFOAM, 2005). Products usually are certified by a third party certification body recognized at international or national level, hence accountable in the case of fraud. Certification is made against the standards of the country where the product is sold. Certified organic food is recognized on the market by the organic label of the certification body (FAO, 2009).

The aim of this research was to determine some important quality parameters and the antioxidant activity of organic and conventional oranges, limes and apples.

## MATERIALS AND METHODS

### Samples

Samples of sweet orange (*Citrus sinensis* L. Osbeck), lime (*Citrus aurantifolia* (Christm) Swingle) and Apple (*Malus domestica* Borkh) were purchased in supermarkets and farmer's market in Rio de Janeiro, Brazil from August to December 2011. Foods were chosen based on Brazil's Food Acquisiton Database 2002/2003 (POF 2002/2003 IBGE) reflecting the fruits most consumed in Brazil. Cultivars used in this study were selected by the organically grown varieties available, since their production is more restricted. All samples were of the same cultivar and had similar sensory characteristics. Certified samples were obtained straight from the refrigerated shelves, except from the conventional and no certified organic samples. Fruits were purchased and immediately sent to the Laboratory of Nutritional Biochemistry of University of State of Rio de Janeiro, Brazil for analyses.

### Sample preparation

All samples were rinsed with tap water to remove adherent contaminants. For the analysis of the ascorbic acid content and antioxidant activity in the samples, determination was done on the same day of purchase to avoid the instability of certain compounds. All fruits were divided into peel and pulp fractions. For each independent analysis, at least 300 g of fruit were put in a commercial juice extractor (Samsom GB- 9001, Greenbison Inc., USA), obtaining a fluid extract which was used in all analyses. All analyzes were determined using five different batches of certified organic, organic (without certification seal) and conventional, each one analysed in triplicate immediately after the fluid extract was obtained.

### Quality parameters

All the analyses were performed according to AOAC (2005). Results were expressed as means±SD where all the analysis was done in triplicate.

The inversion of sucrose was carried out by acid hydrolysis, using 20 ml of the sample heated at 90°C, which received the addition of 1 ml of concentrated HCl and remained in a water bath for 30 min. Then, the mixture was cooled until room temperature and neutralized with sodium hydroxide (NaOH) solution 40%, which was verified using litmus paper strips. Subsequently, the solution was transferred to a 100 ml volumetric flask and the volume was completed with distilled water.

Total soluble solids (°Brix) were measured using a refractometer

at 25°C accurate to two decimals, making readings on two or three drops of juice from the sample. Results were expressed in °Brix. Density was determined with picnometer at the temperature of 25°C. Total titratable acidity was determined by volumetric neutralization using, typically, 0.01 N NaOH and using phenolphthalein as an indicator (pH = 8.1 to 10.0). For lime and orange samples, it was used as a solution of 10% and for apples as a solution of 5%. The total acidity was expressed as the predominant acid in food**.**

Vitamin C (ascorbic acid) was determined by N-Bromosuccamide (NBS) method, where the titration flask initially contains some added potassium iodide (KI). The first small excess of NBS oxidizes some iodide ions to molecular iodine, which then reacts with more iodine (I) - to produce the triiodide ion ($I^3$). The polymeric amylose (starch) molecules wrap themselves around the threaded ions and form a blue colored aggregate to signal the end point. The results were expressed in g/100 g of citric acid.

#### Total antioxidant capacity of 1,1-diphenyl-2-picrylhydrazyl (DPPH)

The antioxidant activity of the samples was subjected to determination of the ability to scavenge the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, according to the method described by Brand-Williams et al. (1995) modified by Miliauskas et al. (2004). Aliquots of sample (5, 10, 15 and 25 µL) were placed in different test tubes containing 3 ml of methanol solution of DPPH. Then, the tubes were left to stand under the light, for 30 min and finally, the absorbance was measured at 515 nm in a spectrophotometer (Turner™ model 340). The ability to scavenge the radical was calculated relating to control (no antioxidant) and expressed as a percentage, according to the following expression:

$$\% \text{ scavenging} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample}) \times 100}{\text{Absorbance of control}}$$

#### Statistical analysis

Data are shown as mean values ± standard deviation of five independent experiments done in triplicate (n = 5). Statistical comparisons were carried out by ANOVA and post hoc Tukey´s test using GraphPad Prism. Differences were considered significant when $p<0.05$.

## RESULTS AND DISCUSSION

### Physico-chemical analysis

#### *Lime (Citrus aurantifolia (Christm) Swingle)*

The results of total titratable acidity were 5.46 ± 0.05 g% for conventional samples and 5.72 ± 0.14 g% for organic samples. Soluble solids were 6.64 ± 0.20 ºBrix and 8.15 ± 0.10 ºBrix for conventional and organic samples, respectively. These results disagree with those observed by Rangel et al. (2011) that evaluated the contents of ascorbic acid, minerals, sugars, total soluble solids, pH, titratable acidity, and juice yield of the acid lime juice, cv.*Tahiti,* from conventional and organic biodynamic production systems. In this study the conventional acid lime showed higher values of titratable acidity and soluble solids. Statistical analyses indicated no significant differences between conventional and certified organic samples for total titratable acidity; however, the soluble solids content of the organic group was higher than that of the conventional group (Table 1).

The soluble solids content and titratable acidity of lime conventional samples presented mean values lower than organic samples. This probably is due to the fact the composition and fruit flavor can be modified by the intensity of sunlight and the use of pesticides (Mattheis and Fellman, 1999).Intense sun rays over the fruit can influence total soluble solids, but not total titratable acidity, but phosphorus fertilization, for instance, may lead to a reduction of total acidity and soluble solids (Rangel et al., 2011).

There were no significant differences ($p > 0.05$) among the values of density and ascorbic acid in lime samples. These results are higher with those observed by Esch et al. (2010) determined by the vitamin C content of conventionally and organically grown fruits by cyclic voltammetry. This study reported values of vitamin C content of 26.2 mg/100 g for conventional and 34.5 mg/100 g for organic lemons.

The vitamin C content in fruit and vegetables can be influenced by some factors such as: genotypic differences, pre-harvest conditions, maturity stage, harvest methods, and post-harvest handling system. In citrus, vitamin C concentration decreases with ripening. However, vitamin C content is higher in mature fruit than in green fruit because the fruit juice volume and fruit size increase in mature fruit. The vitamin C content of citrus juice increases with potassium soil fertilization and decreases with high nitrogen fertilization (Lee et al., 2006).

The total sugar content and °Brix of organic samples differed significantly from the conventional samples. Moreover, this study reported values of vitamin C content of 26.2 mg/100 g for conventional and 34.5 mg/100 g for organic. (Table 1). The results for soluble solids are discordant from those obtained by Rangel et al. (2011), who found higher soluble solids content in fruits from the conventional system.

In general, comparative studies between physical and chemical characteristics of diverse vegetables grown under organic and conventional, have shown variable results, requiring further studies. Therefore, it will be possible to conclusively state whether there are differences in nutritional quality between organic and conventional systems or not (Barret et al., 2007).

#### **Orange (*Citrus sinensis* (L.) *Osbeck*)**

Statistical analyses indicated no significant differences

**Table 1.** Physico-chemical analysis of conventional and organically grown of Lime (*Citrus aurantifolia* (Christm) Swingle)

| Parameter | Sample | Acidity (g%) | Total sugar (g%) | Density (g/cm$^3$) | Brix (º) | Ascorbic Acid (mg%) |
|---|---|---|---|---|---|---|
| Organic | O1 | 5.76 ±0.05 | 2.32 ± 1.05 | 1.215 | 7.95 | 29.26 ± 1.58 |
| | O2 | 6.14 ± 0.19 | 2.39 ± 0.08 | 1.226 | 8.35 | 37.64 ± 1.99 |
| | O3 | 5.44 ± 0.06 | 2.22 ± 0.01 | 1.28 | 7.95 | 48.07 ± 2.49 |
| | O4 | 5.79 ± 0.27 | 2.22 ± 0.05 | 1.01 | 8.35 | 20.95 ± 0.78 |
| | O5 | 5.46 ± 0.13 | 2.32 ± 0.02 | 1.22 | 8.15 | 42.27 ± 3.95 |
| | **Mean ±SD** | **5.72 ± 0.14$^a$** | **2.29 ± 0.24$^a$** | **1.19 ± 0.10$^a$** | **8.15 ± 0.10$^a$** | **35.64 ± 2.16$^a$** |
| Certified Organic | O1 | 5.44 ± 0.06 | 2.34 ± 0.12 | 1.28 | 9.47 | 48.07 ± 2.49 |
| | O2 | 5.79 ± 0.27 | 2.40 ± 0.63 | 1.22 | 8.16 | 46.78 ± 0.39 |
| | O3 | 5.46 ± 0.13 | 2.57 ± 0.08 | 1.01 | 9.47 | 42.27 ± 3.95 |
| | O4 | 5.47 ± 0.06 | 2.48 ± 0.16 | 1.03 | 8.16 | 39.75 ± 1.97 |
| | O5 | 5.46 ± 0.13 | 2.55 ± 1.01 | 1.03 | 9.47 | 50.90 ± 1.57 |
| | **Mean ±SD** | **5.42 ± 0.13$^b$** | **2.47 ± 0.40$^a$** | **1.10 ± 0.13$^a$** | **8.95 ± 0.72$^a$** | **45.55 ± 2.07$^a$** |
| Conventional | O1 | 5.87 ±0.06 | 1.07 ± 0.03 | 1.21 | 6.83 | 27.16 ± 1.45 |
| | O2 | 5.95 ±0.06 | 1.65 ± 0.03 | 1.22 | 6.43 | 36.19 ± 1.95 |
| | O3 | 5.13 ± 0.06 | 1.07 ± 0.03 | 1.11 | 6.68 | 52.53 ± 2.74 |
| | O4 | 4.76 ± 0.02 | 1.65 ± 0.03 | 1.02 | 6.43 | 29.74 ± 0.60 |
| | O5 | 5.57 ± 0.06 | 1.07 ± 0.03 | 1.29 | 6.83 | 51.81 ± 6.40 |
| | **Mean ±SD** | **5.46 ± 0.05$^b$** | **1.30 ± 0.03$^b$** | **1.17 ± 0.11$^a$** | **6.64 ± 0.20$^b$** | **39.49 ± 2.63$^a$** |

Data represent mean ± SD values of triplicate experiments. Tukey–Kramer Multiple Comparison test; Different letters indicate statistically significant differences at the 0.05 level.

between orange samples for density and ascorbic acid (Table 2). Masamba and Nguyen (2008) reported values of 43.4 mg/100 g and 51.8 mg/100 g of vitamin C for conventionally and organically grown oranges, respectively. According to Esch et al. (2010), the levels found was approximately half that amount, showing 27.7 mg/100 g in conventionally grown oranges and 26.2 mg/100 g in organically grown oranges. In this study values of 35.49 mg/100 g in conventionally grown oranges and 39.21 mg/100g in organically grown were detected.

Morillas Ruiz (2005) observed that ecological crops of tangerines and lemons presented higher antioxidant activity and vitamin C content than conventional fruit crops. However, the opposite was observed in oranges, in which the conventional crops had higher vitamin C content than the ecological ones. Worthington (2001) find that the vitamin C content of an organic fruit or vegetable is 27% more, on average, than a comparable conventionally grown fruit or vegetable. In other words, if an average conventional fruit or vegetable contains 100 mg of vitamin C, then a comparable organic one would contain 127 mg.

Total sugar content of organic orange showed higher mean values, when compared to conventional fruit and organic certified. Organic orange and organic certified showed higher mean values of acidity, when compared to conventional fruit. According to Camargo et al. (2011), the fruits cultivars presented the highest percentages in the organic system. For fruits to be well accepted for flavor, there must be a positive correlation among the °Brix and titratable acidity contents (Vangdal, 1985; Fellers, 1991).

### Apple (*Malus domestica* Borkh)

According to statistical analysis there is no significant differences between conventional, certified organic and organic samples for total titratable acidity. According to Amarante et al. (2008), apples organic orchard had lower titratable acidity than fruit from conventional orchard. It can be stated that the fruits rapidly lose acidity with ripening, but in some cases there is a small increase in the values as ripeness advances (Camargo et al., 2011), and this might explain the low variation in acidity among the samples assessed.

No significant difference between samples of apple for density and ascorbic acid. Gazdik et al. (2008) reported apples to have a vitamin C concentration range of 11 to

**Table 2**. Physico-chemical analysis of conventional and organically grown of sweet orange (*Citrus sinensis* L. Osbeck).

| Parameter | Sample | Acidity (g%) | Total sugar (g%) | Density (g/cm$^3$) | Brix (º) | Ascorbic Acid (mg%) |
|---|---|---|---|---|---|---|
| Organic | O1 | 0.60 ± 0.02 | 15.55 ±1.73 | 1.03 | 10.12 | 50.85±1.86 |
| | O2 | 0.95 ±0.02 | 15.55 ±1.73 | 1.03 | 10.73 | 33.44±2.79 |
| | O3 | 0.90 ± 0.05 | 12.85 ±0.00 | 1.04 | 8.15 | 32.65±1.13 |
| | O4 | 0.42 ± 0.04 | 17.13 ± 0.00 | 1.05 | 10.12 | 39.92±0.91 |
| | O5 | 0.42 ± 0.04 | 9.83 ± 0.00 | 1.01 | 10.73 | 38.81±0.68 |
| | Mean ±SD | **0.66 ± 0.03$^a$** | **14.18 ± 0.69$^a$** | **1.03 ± 0.01$^a$** | **9.97 ± 1.06$^a$** | **39.13±1.47$^a$** |
| Certified Organic | O1 | 0.96 ± 0.01 | 7.47 ± 0.69 | 1.05 | 10.73 | 49.28±3.83 |
| | O2 | 0.68 ± 0.02 | 10.46 ±0.31 | 1.04 | 10.11 | 38.24±1.39 |
| | O3 | 0.62 ± 0.02 | 12.09 ± 0.00 | 1.03 | 9.46 | 33.64+0.91 |
| | O4 | 0.45 ± 0.01 | 9.67 ± 0.00 | 1.03 | 8.15 | 35.49±1.52 |
| | O5 | 0.68 ± 002 | 5.91 ± 0.13 | 1.05 | 8.81 | 39.8±1.34 |
| | Mean±SD | **0.69 ± 0.01$^a$** | **9.12 ± 0.23$^b$** | **1.04 ±0.01$^a$** | **9.45 ±1.02$^a$** | **39.29±1.79$^a$** |
| Conventional | O1 | 0.63 ± 0.04 | 5.16 ± 0.42 | 1.05 | 10.73 | 43.88±1.86 |
| | O2 | 0.76 ± 0.01 | 8.57 ± 0.36 | 1.02 | 10.12 | 45.68±0.92 |
| | O3 | 0.96 ± 0.01 | 7.47 ± 0.69 | 1.05 | 10.73 | 49.28±3.83 |
| | O4 | 0.68 ± 0.02 | 10.46 ± 0.31 | 1.04 | 10.11 | 38.24±1.39 |
| | O5 | 0.62 ± 0.02 | 12.09 ± 0.00 | 1.03 | 9.46 | 33.64+0.91 |
| | Mean ±SD | **0.45 ± 0.01$^b$** | **9.67 ± 0.00$^b$** | **1.03 ± 0.02$^a$** | **10.23 ± 0.52$^a$** | **35.49±1.52$^a$** |

Data represent mean ± SD values of triplicate experiments. Tukey–Kramer Multiple Comparison test; Different letters indicate statistically significant differences at the 0.05 level.

19 mg/100 g. This is not comparable to our results which showed the average value of organic apples to be 39.14 mg/100 g and 26.58 mg/100 g for conventional apples. According to Esch et al. (2010), results found in the current literature regarding the ascorbic acid present in conventional and organic fruit are ambiguous. Other external factors such as the conditions of storage and transport, has considerable influence on the vitamin C content of fruit.

The total sugar content of most cultivars ranged between 24.67 and 17.90 g/100 g, but cultivars from organic growing reached higher values (Table 3). The acid content of organic and conventional cultivars were similar. The higher sugar content does not automatically mean sweeter taste of apples, because the amount of organic acids is also important for perceiving the sweetness (Veberic and Stampar, 2005).

Total soluble solids (°Brix) represent a group of substances present in apple juice, with a predominance of sugars, which are directly related to the density. In addition, other factors affect the production of sugar in the apple juice, especially the variables participating in photosynthesis as heat intensity, solar radiation and soil moisture. So warm climates during maturation apple favor production of sugar and therefore soluble solids (RIzzon et al., 2005).

## Antioxidant activity

Regarding the antioxidant activity (Figure 1) there was an increase in the percentage of reduction of DPPH in samples of organic lime and oranges compared to conventional, with no statistical difference ($p > 0.05$) between no certified organic and certified organic fruit. Regarding the apple, conventional samples showed a higher percentage of reduction of DPPH. According to Tarozzi et al. (2005), the protective effect against oxidative damage was higher for organic oranges, probably due to higher levels of phenolic compounds, anthocyanins and ascorbic acid. Thus, it is recommended additional studies to confirm that the practice of organic farming is favorable to increase antioxidant activity of other varieties of fruits and vegetables. Blueberries produced from organic culture contained significantly higher amounts of phytonutrients than those produced from conventional culture (Wang et al., 2008).

Increase of phenolics in fruits of organic production was noticed in various fruit species. For example, there was a parallel increase in polyphenol content of organic

**Table 3.** Physico-chemical analysis of conventional and organically grown of Apple (*Malus domestica* Borkh).

| Parameter | Sample | Acidity (g%) | Total sugar (g%) | Density (g/cm$^3$) | Brix (º) | Ascorbic Acid (mg%) |
|---|---|---|---|---|---|---|
| Organic | O1 | 0.32±0.03 | 26.33±4.10 | 1.05 | 14.581 | 62.37±7,44 |
| | O2 | 0,32±0,02 | 27,23±1,18 | 1,01 | 13,953 | 35,21±3,10 |
| | O3 | 0.26±0.01 | 24.26±0.89 | 1.05 | 13.953 | 69.01±9.14 |
| | O4 | 0.26±0.01 | 20.69±1.15 | 1.01 | 8.155 | 45.73±4.65 |
| | O5 | 0.28±0.00 | 24.85±1.66 | 1.01 | 8.155 | 39.82±3.56 |
| | **Mean ±SD** | **0.29±0.01[a]** | **24.67±1.79[a]** | **1.02±0.02[a]** | **11.75±3.30[a]** | **39.14±5.57[a]** |
| Certified Organic | O1 | 0.39±0.01 | 11.48±2.18 | 1.1 | 15.207 | 64.38±4.33 |
| | O2 | 0.35±0.00 | 22.38±0.96 | 1.05 | 15.207 | 18.04±3.67 |
| | O3 | 0.34±0.00 | 24.81±1.13 | 1.04 | 11.407 | 17.48±2.23 |
| | O4 | 0.21±0.01 | 18.81±1.71 | 1.04 | 12.048 | 42.88±5.79 |
| | O5 | 0.29±0.00 | 17.41±3.27 | 1.01 | 12.686 | 25.58±2.42 |
| | **Mean ±SD** | **0.32±0.00[a]** | **19.97±1.85[a]** | **1.04±0.03[a]** | **13.31±1.78[a]** | **33.67±3.68[a]** |
| Conventional | O1 | 0.21±0.00 | 11.48±2.18 | 1.1 | 15.207 | 59.88±1.57 |
| | O2 | 0.26±0.00 | 23.60±1.82 | 1.04 | 11.407 | 14.23±2.02 |
| | O3 | 0.35±0.01 | 18.81±1.71 | 1.05 | 13.953 | 18.40±1.33 |
| | O4 | 0.33±0.02 | 18.10±1.96 | 1.03 | 12.686 | 22.15±1.81 |
| | O5 | 0.38±0.00 | 17.54±2.59 | 1.02 | 15.829 | 18.25±2.53 |
| | **Mean ±SD** | **0.30±0.00[a]** | **17.90±2.05[a]** | **1.04±0.03[a]** | **13.81±1.80[a]** | **26.58±1.85[a]** |

Data represent mean ± SD values of triplicate experiments. Tukey–Kramer Multiple Comparison test; Different letters indicate statistically significant differences at the 0.05 level.

peaches and pears when compared to the corresponding conventional samples (Carbonaro et al., 2002). Veberic and Stampar (2005) observed that cultivars of organic production showed significantly higher contents of total phenolic compounds analyzed in the apple pulp compared to the apples of integrated production and resistant cultivars.

Organically, grown cultivars also showed higher contents of total phenolics in apple peel, although there were no significant differences between organically grown and intensively grown cultivars. The higher content of phenolics in apple pulp and peel is not only the consequence of the fact that organically grown apples are more exposed to biotic and abiotic stressors (diseases, pests, lack of mineral nutrients), but it appears also to be due to the selection of modern cultivars towards less tough fruits with not such astringent taste. The consumers prefer fruits with gentler taste (Lattanzio, 2003), which leads to choosing those modern cultivars that exhibit lower contents of some groups of phenolics.

## Conclusion

Although the literature suggests differences in content of nutrients and other nutritionally relevant substances (nutrients and other substances) in organically and conventionally production, comparing fruits from both types of agriculture, few statistical differences were observed in acidity and total sugar for sweet orange (*Citrus sinensis* L. Osbeck) and lime (*Citrus aurantifolia* (Christm) Swingle). The differences detected in content of nutrients and other substances between organically and conventionally produced crops and livestock products are biologically plausible and most likely relate to differences in crop and soil quality. However, antioxidant capacity tended to be higher in plants grown using organic agriculture, with higher concentrations in the plant fractions most exposed to the environment. The literature suggests that the exposure of plant foods to stressful situations could modulate the synthesis of defense substances such as polyphenols, that reflects in a higher antioxidant capacity. However, the results showed distinct profiles according to the plant food analysed, suggesting that modulation of these parameters due to alterations in the cultivation practice is unique to the specific species and cultivar of vegetables.

## Authors' contributions

CBDS and RLS, performed experiments and summarized

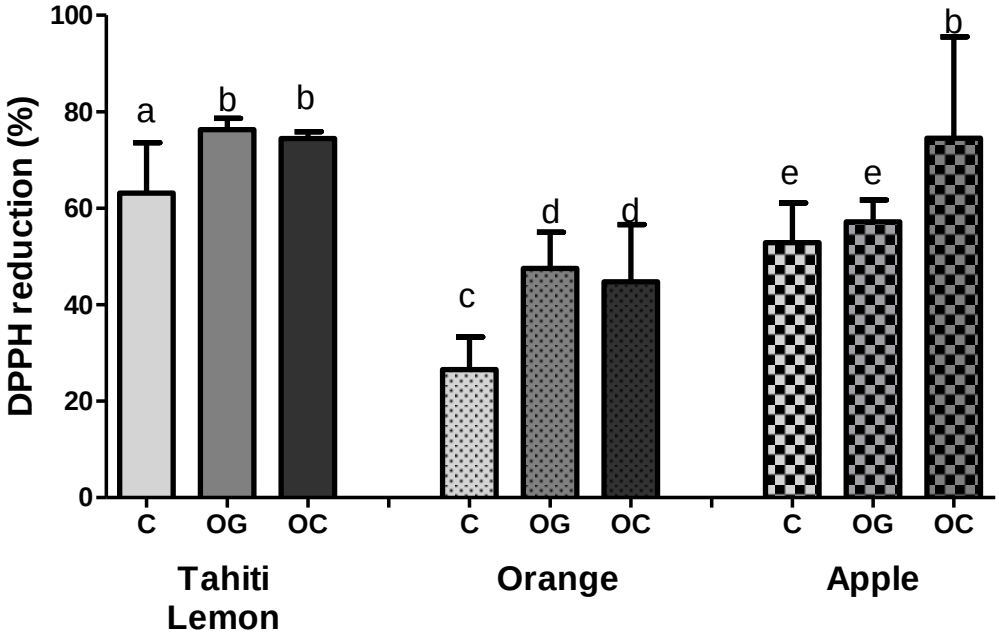


**Figure 1.** Comparison of the antioxidant activity of Tahiti lemon, orange and apple conventional (C), organic (OG) and certified organic (OC). Tukey–Kramer Multiple Comparison test; Different letters indicate statistically significant differences at the 0.05 level.

the data; TAJ, BDS, RLS and MSEM designed experiments; TAJ, BDS and RLS, MSEM, wrote the paper; all authors have read and approved the final manuscript.

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*Full Length Research Paper*

# *In vitro* antimicrobial potential of organic solvent extracts of novel actinomycetes isolated from forest soil

**M. Valan Arasu[1], T.S.Rejiniemon[2], N. A. Al-Dhabi[1], V. Duraipandiyan[1], P. Agastian[3*], V.A.J. Huxley[2], C.E. Song [4] and K.C Choi [5*]**

[1]Department of Botany and Microbiology, Addiriyah Chair for Environmental Studies, College of Science, King Saud University, Riyadh 11451, Saudi Arabia.
[2]Department of Zoology, Thiru Vika Government Arts College, Thiruvarur, 610 003, Tamil Nadu, India.
[3]Department of Plant Biology and Biotechnology, Loyola College, Chennai, 600 034, India.
[4]Lifelong Education Center, Chonnam National University, Kwangju, 500-757, Republic of Korea
[5]Grassland and forage division, National Institute of Animal Science, RDA, Seonghwan-Eup, Cheonan-Si, Chungnam, 330-801, Republic of Korea.

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***In vitro*** **screening of antibacterial and antifungal activities of hexane, chloroform, ethyl acetate, methanol and water extracts of selected promising actinomycetes strains were studied towards Gram-positive, Gram-negative bacteria, dermatophytes and opportunistic pathogens. Crude antimicrobial metabolites were extracted by liquid-liquid extraction and solid-liquid extraction method using hexane, chloroform, ethyl acetate and methanol. The lowest minimum inhibitory concentration (MIC) of the extracts was assessed by the broth micro dilution method. All the extracts obtained from eight strains showed promising activity against tested Gram-positive bacteria. The hexane extracts of strain ERI-1 exhibited activity against *Bacillus subtilis, Staphylococcus aureus* and *Staphylococcus epidermidis* at a concentration of 5 mg/ml. Ethyl acetate extract of strain ERI-4 showed MIC of 5 mg/ml but *S. epidermidis* and *S. aureus. B. subtilis* exhibited activity at 2.5 mg/ml. However, ethyl acetate and lyophilized water extract of strain ERI-3 inhibited the growth of *Trichophyton mentagrophytes, Trichophyton rubrum, Epidermophyton floccosum* and *Scropulariopsis* sp at 10 mg/ml. Overall significant antibacterial and antifungal activities were noted in the ethyl acetate extract of ERI-1 and ERI-3. Methanol extract of ERI-26 exhibited good antibacterial and antifungal activities.**

**Key words:** Actinomycetes, organic extracts, minimum inhibitory concentration (MIC), antimicrobial activity.

## INTRODUCTION

Actinomycetes are Gram-positive bacteria showing a filamentous growth. Actinomycetes are a group of organisms widespread in nature, and play a significant role in the future of biotechnology, because of their importance as producers of vitamins, enzymes, antitumour agents, immune modifying agents and, mainly,

antibiotic compounds (Sofia and Boldi, 2006). According to Maya et al. (2011) number of novel molecules from actinomycetes were discovered. Streptomycetes, the Gram positive filamentous bacteria are widely distributed in a variety of natural and man-made environments, constituting a significant component of the microbial population in most soils. The results of extensive screenings have led to the discovery of about 4,000 antibiotic substances from bacteria and fungi, many of which have been applied in human medicine, veterinary medicine and agriculture (Adinarayana et al., 2007). Most of metabolites are produced from *Streptomyces*. Approximately 75% of metabolites originated from *Streptomyces* genus and at least 5000 documented bioactive compounds are known as being produced by *Streptomyces* genus (Vasanthabharathi et al., 2011). Most *Streptomyces* are used in the production of a diverse array of antibiotics including aminoglycosides, macrolides, β-lactams, peptides, polyenes, polyether, tetracyclines, etc (Augustine et al., 2005). In searching for new antibiotics, number of different bacteria, actinomycetes, Streptomycetes, fungi and algae have been investigated. To prevent exponential emergence of microorganisms from becoming resistant to the clinically available antibiotics already marketed, a periodic replacement of the existing antibiotics is necessary. In the present study, extraction of antimicrobial metabolites from *Streptomyces* and its antimicrobial effects on bacteria and fungi were studied. Previously, nutrients required for the antimicrobial compounds production were optimized under the shake flask condition for the identified *Streptomyces* strains (Arasu et al., 2012; 2013). This study focused on the extraction of antimicrobial metabolites by using different solvents and evaluates minimum inhibitory concentration by broth micro dilution method.

## MATERIALS AND METHODS

### Chemicals and solvents

Glucose and all other chemicals were obtained from Himedia (India). Organic solvents were procured from Himedia (India).

### Antimicrobial compound extraction

#### *Liquid - liquid extraction*

Actinomycetes were grown in modified nutrient glucose broth medium for six days. After incubation, the fermentation medium was collected and filtered through Whatman No.1 filter paper. The total culture filtrate 4000 ml was used for the series of solvent extraction by using hexane, chloroform, ethyl acetate and methanol. The low polar to high polar solvent was selected for the organic solvent extraction. Three folds volume of the solvent was mixed thoroughly with the broth by shaking them in 1000 ml capacity separating funnel and allowed to stand for 1 h. The solvents were removed using simple distillation and vacuum rotary evaporator at 40ºC. The extracts were stored at 4ºC until further use. Water extract was lyophilized and the concentrated metabolites were stored at 4ºC until further use.

#### *Solid - liquid extraction*

A metabolite which could not extract from fermented broth was extracted by this method. The spore suspensions of the culture were inoculated on Modified Nutrient Glucose Agar (MNGA) media and incubated at 28ºC for six days. Then, agar media with cultures were taken into a 500 ml flask containing 100 ml of methanol and kept in shaker for 2 h at 200 rpm. Then the suspension was centrifuged at 8000 rpm, 10 min to separate the organic phase and extracted twice. The methanol phase was concentrated by using vaccum at $35^0$ C. The extracts were stored at 4ºC until further use.

### Test organisms

The reference strains used in this study was procured from American type culture collection (ATCC) and Microbial type culture collection Chandigarh, India (MTCC). The following microorganisms were used to test minimum inhibitory concentration of the extracts: Gram-positive (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* MTCC 3615, *Bacillus subtilis* MTCC 441 and *Enterococcus faecalis* ATCC 29212) and Gram-negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 15380, *Proteus vulgaris* MTCC 1771, *Erwinia* sp MTCC 2760, *Vibrio fischer*i MTCC 1738 and *Salmonella typhi* MTCC 733).

### Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was performed according to the standard reference method (NCCLS, 1999). The extracts were dissolved in water + 2% dimethyl sulfoxide (DMSO). The initial concentration of extract was 10 to 0.156 mg/ml. The initial test concentration was serially diluted twofold. Each well was inoculated with 5 µl of suspension containing $10^8$ CFU/ml of bacteria. The antibacterial agent streptomycin was included in the assays as positive controls. The plates were incubated 24 h at 37°C. After incubation 5 µl of testing broth was placed on the sterile MHA plates for bacteria and incubated at respective temperature. The MIC for bacteria was determined as the lowest concentration of the extracts inhibiting the visual growth of the test cultures on the agar plate. Three replications were maintained.

### Fungal strains

The fungal strains were procured from Microbial type culture collection Chandigarh, India (MTCC), and Christian Medical College, Vellore, India. The following fungi were used for experiments: *Trichophyton rubrum* MTCC 296, *T. rubrum* 57/01, *T.*

*Corresponding authors. E-mail: past_hod@rediffmail.com, choiwh@korea.kr..

*mentagrophytes* 66/01, *T. simii* 110/02, *Epidermophyton floccosum* 73/01, *Scopulariopsis* sp. 101/01 *Aspergillus niger* MTCC 1344, *Botyritis cinerea, Curvularia lunata* 46/01, and *Candida albicans* MTCC 227.

#### Preparation of fungal spore

The filamentous fungi were grown on Sabouraud Dextrose Agar (SDA) slants at 28°C for 10 days and the spores were collected using sterile doubled distilled water and homogenized. Yeast was grown on Sabouraud Dextrose Broth (SDB) at 28°C for 48 h.

#### Antifungal assays

The antifungal activity was performed according to the standard reference method (NCCLS, 1999). The extracts were dissolved in water+2% dimethyl sulfoxide (DMSO). The initial concentration of extract was 10 mg/ml. The initial test concentration was serially diluted twofold. Each well was inoculated with 5 µl of suspension containing $10^4$ spore/ml of fungi. The antifungal agent Fluconazole was included in the assays as positive controls; the plates were incubated for 24 h up to 9 days at 27°C for dermatophytic strains. MIC was defined as the lowest extract concentration, showing no visible fungal growth after incubation time. Experiment was carried out in triplicates.

## RESULTS

Five different extracts from eight strains of actinomycetes were screened against four Gram-positive and eight Gram-negative bacteria; among them three strains extracts exhibited good activity against tested microbes (Table 1). Some extract had a significant activity for Gram-positive bacteria but not on Gram-negative bacteria. Ethyl acetate extracts of all the strains showed activity against more than four different bacteria.

### Activity against Gram-positive bacteria

All the extracts from eight strains showed activity against tested Gram positive bacteria. The hexane extracts of ERI-1 exhibited activity against *B. subtilis, S. aureus* and *S. epidermidis* at a concentration of 5 mg/ml. Chloroform and methanol extract did not show antibacterial activity. Lyophilized water extract showed MIC of 5 mg/ml for *S. aureus, S. epidermidis* and *E. faecalis*. *B. subtilis* exhibited MIC of 2.5 mg/ml. Ethyl acetate extract showed MIC of 5 mg/ml to *B. subtilis, S. aureus, S. epidermidis* and *E. faecalis*. Hexane extracts of ERI-3 showed MIC of 5 mg/ml to *S. epidermidis* and *B. subtilis* for 10 mg/ml. Chloroform extract did not exhibit any activity against Gram positive bacteria. Ethyl acetate extract showed MIC of 10 mg/ml for *B. subtilis, S. aureus* and *E. faecalis*. For *S. epidermidis* growth was inhibited at a concentration of 2.5 mg/ml. Lyophilized water extract exhibited MIC of 5 mg/ml to *B. subtilis* and *S. aureus. E. faecalis* and *S. epidermidis* revealed MIC of 10 and 2.5 mg/ml respectively in the lyophilized water extract.

Hexane and ethyl acetate of ERI-26 did not show any activity against both Gram positive and Gram negative bacteria. The chloroform extract showed MIC of 10 mg/ml to all the tested bacteria. Methanol extract exhibited MIC of 2.5 mg/ml to *B. subtilis, S. epidermidis* and *E. faecalis.* Lyophilized water extract exhibited MIC of 5 mg/ml to *E. faecalis* and *B. subtilis. S. epidermidis* and *S. aureus* showed MIC of 10 mg/ml. Ethyl acetate extract of ERI-4 showed MIC of 5 mg/ml to *S. epidermidis* and *S. aureus. B. subtilis* exhibited MIC of 2.5 mg/ml for ethyl acetate extract and *S. aureus* revealed 10 mg/ml. Hexane, methanol and lyophilized water extract did not show any activity. Chloroform extracts of ATSI-13 showed MIC of 10 mg/ml to all the tested bacteria. Ethyl acetate extract exhibited MIC of 5 mg/ml. Hexane, methanol and lyophilized water extract of ATSI-13 did not show any activity against bacteria (Table 1).

### Activity against Gram-negative bacteria

Most of the actinomycetes extracts did not inhibit the growth of Gram negative bacteria. Ethyl acetate extract of ERI-1 inhibited the growth of *P. aeruginosa.* Hexane, ethyl acetate and lyophilized water extract showed MIC of 5 mg/ml to *E. coli. P. aeruginosa, K. pneumoniae, Xanthomonas* sp, *Erwinia* showed MIC of 10 mg/ml to ethyl acetate and lyophilized water extracts. Ethyl acetate extract showed MIC of 10 mg/ml to *S.typhi* and *V. fischeri*. Hexane, chloroform, ethyl acetate and lyophilized water extract did not show any activity against *P. vulgaris.* Lyophilized water extracts of ERI-3 showed MIC of 10 mg/ml to all the tested bacteria. Ethyl acetate extract also showed MIC of 10 mg/ml to all the tested bacteria except *S. typhi, V. fischeri* and *P.vulgaris.* Hexane and chloroform extract of ERI-3 did not show any antibacterial activity. The methanol extract of ERI-26 exhibited MIC of 5 mg/ml to *E. coli, P. aeruginosa* and *K. pneumoniae. Xanthomonas* sp., *Erwinia, S. typhi, V. fischer*i, *P. vulgaris* showed MIC of 10 mg/ml. Lyophilized water extract showed MIC of 5 mg/ml to *E.coli.* Hexane, chloroform and ethyl acetate extracts of ERI-26 did not reveal any activity against tested bacteria.

### Antifungal activity

Results of antifungal activity were summarized in Table 2. All the 8 strains showed varying degrees of antifungal activity. Most of the extracts inhibited more than four fungal strains. From the evaluation we found that ethyl acetate extracts inhibited the growth of fungus. Hexane and methanol extracts also nearly showed the same level

**Table 1.** Minimum inhibitory concentration of crude extracts obtained from selected actinomycetes against bacteria.

| Strain | Extract | MIC (mg/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ***B. s*** | ***S. a*** | ***S. e*** | ***E. f*** | ***E. c*** | ***P. a*** | ***K. p*** | ***X*** | ***Er.*** | ***S. t*** | ***V. f*** | ***P. v*** |
| | He | 5 | 5 | 5 | 10 | 5 | - | - | - | - | - | - | - |
| | Cl | - | - | - | - | - | - | - | - | - | - | - | - |
| ERI-1 | Ea | 5 | 5 | 2.5 | 5 | 5 | 10 | 10 | 10 | 10 | 10 | 10 | - |
| | Me | - | - | - | - | - | - | - | - | - | - | - | |
| | Ly | 2.5 | 5 | 5 | 5 | 5 | 10 | 10 | 10 | 10 | - | - | - |
| | He | 10 | 10 | 5 | - | - | - | - | - | - | - | - | - |
| | Cl | - | - | - | - | - | - | - | - | - | - | - | - |
| ERI-3 | Ea | 10 | 10 | 5 | 10 | 10 | 10 | 10 | 10 | 10 | - | - | - |
| | Ly | 5 | 5 | 2.5 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | - |
| | He | - | - | - | - | - | - | - | - | - | - | - | - |
| | Cl | 10 | 10 | 10 | 10 | - | - | - | - | - | - | - | - |
| ERI-26 | Ea | - | - | - | - | - | - | - | - | - | - | - | - |
| | Me | 2.5 | 5 | 2.5 | 2.5 | 5 | 5 | 5 | 10 | 10 | 10 | 10 | 10 |
| | Ly | 5 | 10 | 10 | 5 | 5 | 10 | 10 | 10 | 10 | 10 | - | - |
| | He | - | - | - | - | - | - | - | - | - | - | - | - |
| | Cl | 10 | - | - | - | - | 10 | 10 | 10 | - | - | - | - |
| ERI-4 | Ea | 2.5 | 10 | 5 | 5 | 10 | 5 | 10 | 10 | - | - | - | - |
| | Me | - | - | - | - | - | - | - | - | - | - | - | - |
| | Ly | - | - | - | - | - | - | - | - | - | - | - | - |
| | He | - | - | - | - | - | - | - | - | - | - | - | - |
| | Cl | 10 | 10 | 10 | - | - | - | - | - | - | - | - | - |
| ATS1-13 | Ea | 5 | 5 | 5 | 5 | - | - | - | - | - | - | - | - |
| | Me | - | - | - | - | - | - | - | - | - | - | - | - |
| | Ly | - | - | - | - | - | - | - | - | - | - | - | - |
| | He | 5 | 5 | 5 | 2.5 | - | - | - | - | - | - | - | - |
| | Cl | - | - | 5 | 5 | - | - | - | - | - | - | - | - |
| AMW-17 | Ea | 5 | 5 | 2.5 | 5 | 10 | - | - | - | - | - | - | - |
| | Me | - | - | - | - | - | - | - | - | - | - | - | - |
| | Ly | 2 | 20 | 2.5 | - | - | - | - | - | - | - | - | - |
| | He | 10 | 10 | 5 | 10 | 10 | - | - | - | - | - | - | - |
| | Cl | - | - | - | - | - | - | - | - | - | - | - | - |
| BMS-4 | Ea | - | - | - | - | - | - | - | - | - | - | - | - |
| | Me | - | - | - | - | - | - | - | - | - | - | - | - |
| | Ly | 10 | 10 | 10 | 10 | 10 | - | - | - | - | - | - | - |
| | He | 5 | 5 | 2.5 | 5 | 10 | 10 | 10 | 10 | 10 | - | 10 | - |
| | Cl | - | - | - | - | - | - | - | - | - | - | - | - |
| AMW-23 | Ea | 5 | 5 | 10 | 10 | - | - | 10 | 10 | 10 | - | - | - |
| | Me | - | - | - | - | - | - | - | - | - | - | - | - |
| | Ly | 5 | 5 | 5 | 5 | 5 | 10 | 10 | - | - | - | - | - |

He - Hexane; Cl -Chloroform, Ea -Ethyl acetate; Me -Methanol, Ly - Lypholized water extract *B.s - B. subtilis; S.a - S. aureus ;S.e - S. epidermidis ;E.f - E. faecalis; E.c - E.coli*; *P.a - P. aeruginosa; K.p - K. pneumoniae; X.sp - Xanthomonas* sp.; *E.*sp - *Erwinia* sp;*S.t - S.typhi* ; *V.f - V. fischer*i ;*P.v - P. Vulgaris.*

**Table 2.** Minimum inhibitory concentration of crude extracts obtained from selected actinomycetes against fungi.

| Strain | Extract | MIC (mg/ml) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ***T. m*** | ***E. f*** | ***T. s*** | ***C. l*** | ***A. n*** | ***B. c*** | ***T. r 296*** | ***T. r 57*** | ***Scro*** | ***C. a*** |
| | He | - | - | - | 10 | 5 | 10 | - | - | - | 5 |
| | Cl | - | - | - | - | - | - | - | - | - | - |
| ERI-1 | Ea | 10 | 10 | 10 | 10 | 2.5 | 10 | 10 | 10 | 10 | 2.5 |
| | Me | 10 | 10 | 10 | 10 | 10 | 5 | - | - | - | 5 |
| | Ly | 10 | 10 | 10 | 5 | 5 | 5 | 10 | 10 | - | 2.5 |
| | He | 10 | - | - | 10 | 10 | - | - | - | - | 10 |
| | Cl | - | - | - | - | - | - | - | - | - | - |
| ERI-3 | Ea | 10 | 10 | 10 | 5 | 2.5 | 10 | 10 | 10 | 10 | 5 |
| | Ly | 10 | 10 | 5 | 5 | 5 | 5 | 10 | 10 | 10 | 10 |
| | He | - | - | - | - | - | - | - | - | - | - |
| | Cl | - | - | - | - | - | - | - | - | - | - |
| ERI-26 | Ea | 10 | 10 | 10 | 10 | - | - | - | - | - | - |
| | Me | 10 | 10 | 10 | 5 | 2.5 | 10 | 10 | 10 | 10 | 1.25 |
| | Ly | 10 | 5 | 10 | 10 | 10 | - | - | - | - | - |
| | He | - | - | - | - | - | - | - | - | - | 10 |
| | Cl | - | - | - | - | - | - | - | - | - | 10 |
| ERI-4 | Ea | - | - | - | - | - | - | - | - | - | 5 |
| | Me | - | - | - | - | - | - | - | - | - | 10 |
| | Ly | 10 | 10 | 10 | 5 | 5 | 5 | 10 | 10 | 10 | 5 |
| | He | - | - | - | - | - | - | - | - | - | - |
| | Cl | - | - | - | - | - | - | - | - | - | - |
| ATS1-13 | Ea | - | - | - | - | - | - | - | - | - | - |
| | Me | - | - | - | - | - | - | - | - | - | - |
| | Ly | - | - | - | - | - | - | - | - | - | - |
| | He | - | - | - | - | - | - | - | - | - | |
| | Cl | - | - | - | - | - | - | - | - | - | - |
| AMW-17 | Ea | 10 | 10 | 10 | 10 | 10 | - | - | 10 | 10 | 5 |
| | Me | - | - | - | - | - | - | - | - | - | - |
| | Ly | 10 | 10 | - | 10 | 5 | 10 | - | - | - | 5 |
| | He | - | - | - | - | - | - | - | - | - | - |
| | Cl | - | - | - | - | - | - | - | - | - | - |
| BMS-4 | Ea | - | - | - | - | - | - | - | - | - | - |
| | Me | - | - | - | - | - | - | - | - | - | - |
| | Ly | 10 | 10 | - | 10 | 10 | 10 | - | - | - | 10 |
| | He | - | - | - | - | - | - | - | - | - | - |
| | Cl | 10 | - | - | 10 | 5 | 5 | - | - | - | 5 |
| AMW-23 | Ea | 10 | 10 | - | 10 | 10 | 10 | - | - | - | 10 |
| | Me | - | - | - | - | - | - | - | - | - | - |
| | Ly | 10 | 10 | 10 | 10 | 5 | - | - | - | - | 5 |

He -Hexane; Cl -Chloroform, Ea - Ethyl acetate; Me - Methanol, Ly - Lypholized water extract. *T.m - T. mentagrophytes*; *E.f - Epidermophyton floccosum*, T.s. - *T. simii; C.l - Curvularia lunata*, *A.n - Aspergillus niger* ; *B.c -Botrytis cinerea; T.r- Trichophyton rubrum*, Scro - *Scropulariopsis* sp.; *C.a- Candida albicans* MTCC 227.

of inhibition against fungal growth. Chloroform extracts showed minimum antifungal activity. Ethyl acetate, methanol and lyophilized water extracts of ERI-1 inhibited the growth of *T. rubrum, T. mentagrophytes* and *T. simii* at MIC values of 10 mg/ml. In addition ethyl acetate and lyophilized water extract inhibited growth of *T. rubrum* (296, 57), *B. cinerea* and *Scropulariopsis* sp. at 10 mg/ml and *C. albicans*, *A. niger* at 2.5 mg/ml. Methanol and lyophilized water extract inhibited the growth of *B. cinerea* at MIC of 5 mg/ml and *C. albicans* showed MIC of 2.5 mg/ml. Hexane extract of ERI-1 inhibited the growth of *A. niger* and *C. albicans* at MIC of 5 mg/ml. Chloroform extract did not show antifungal activity against tested fungi. Hexane extract inhibited the growth of *C. lunata* and *B. cinerea* at MIC of 10 mg/ml also *A. niger* growth was inhibited at 5 mg/ml concentration (Table 2).

Ethyl acetate and lyophilized water extract of ERI-3 inhibited the growth of *T. mentagrophytes T. rubrum, E. floccosum* and *Scropulariopsis* sp at MIC of 10 mg/ml. Ethyl acetate extract of ERI-3 showed MIC of 2.5 mg/ml to *A. niger* and also 5 mg/ml to *C. albicans.* Hexane extract inhibited the growth of *T. rubrum, C. lunata, C. albicans* and *A .niger* with MIC of 10 mg/ml. Lyophilized water extract revealed MIC of 10 mg/ml to *T. simii, C. lunata, A. niger* and *B. cinerea.* Chloroform extract of ERI-3 did not show activity against tested fungi. The methanol extract of ERI-26 showed activity against all the tested fungi. *C. albicans* growth was inhibited by methanol extract of ERI-26 with MIC of 2.5 mg/ml followed by *A. niger* at 5 mg/ml. *C. lunata* growth was inhibited at 5 mg/ml. *T. rubrum, T. mentagrophytes, T. simii, B. cinerea, T. rubrum* (296, 57) and *Scropulariopsis* sp growth were inhibited at 10 mg/ml. Hexane and chloroform extracts of ERI-26 did not inhibit the growth of tested fungi. Minimum Inhibitory Concentration of all the solvent extracts obtained from ERI-4 recorded as10 mg/ml for *C. albicans* except ethyl acetate. Lyophilized water extract showed MIC of 10 mg/ml for *T. rubrum, E. floccosum, T. simii, T. mentagrophytes* and *Scropulariopsis* sp*. C. albicans*, *C. lunata, A. niger* and *B. cinerea* showed MIC at 5 mg/ml. None of the tested fungus was inhibited by extracts obtained from ATS1-13. Hexane and chloroform extracts of AMW-17 did not show activity against tested fungi. Ethyl acetate and lyophilized water extract exhibited MIC of 10 mg/ml to *T. rubrum, C. lunata*, *B. cinerea* and *T. mentagrophytes.* Ethyl acetate extract inhibited the growth of *T. rubrum* (57) and *Scropulariopsis* sp at MIC of 10 mg/ml. Lyophilized water extract recorded MIC of 5 mg/ml to *A. niger* and *C. albicans.*

## DISCUSSION

In the present study, five different extracts of eight actinomycetes were tested for antimicrobial activity. All the isolates were grown in this medium for 96 to 120 h at 30$^{o}$C for antimicrobial compound production. The spent medium was used for extraction of antimicrobial metabolites. Forar et al. (2008) used sucrose, $(NH_4)_2SO_4$ and yeast extract as a fermentation medium for new actinomycete strain SK4-6. This organism exhibited strong activity against bacteria including methicillin resistant *S. aureus* and *M. luteus*, in addition to the causative agents of Candidiasis and Aspergillosis diseases.

Hexane extract of ERI-1, ERI-3, AMW-17, BMS-4 and AMW-23 showed antibacterial activity against Gram-positive bacteria and Gram-negative bacteria. Hexane extract of ERI-1 showed MIC of 5 mg/ml for *B. subtilis*, *S. aureus* and *S. epidermidis.* Hexane extract of ERI-3 showed MIC of 5 mg/ml for *S. aureus.* However Forar et al. (2007) used a range of solvents like petroleum ether, n-hexane, chloroform, diethyl ether, ethyl acetate, butyl acetate, benzene, n-butanol and ethanol to extract the antimicrobial metabolite from actinomycetes strain RAF-10. They reported that n-hexane, chloroform and diethyl ether were poor solvent for antibiotic extraction and n-butanol was good for the extraction of active compounds. Sumitha and Philip (2006) extracted antimicrobial metabolite from actinomycetes by ethyl acetate, Hexane and 1-butanol. The extracts were found to be active against *Vibrios*. Hexane extract of S26 showed inhibition against *V. alginolyticus*. Chloroform extract of ERI-26 showed activity against *B. subtilis, S. aureus, S. epidermidis* and *E. faecalis*. Moustafa et al. (2006) isolated meroparamycin from the fermented broth of *Streptomyces* sp. by chloroform as solvent. Jaime et al. (1991) and Thangadurai et al. (2004) used ethyl acetate, chloroform and hexane for the extraction of antimicrobial metabolites from bacteria. Three organic solvents of differing polarity were used subsequently to extract the active principle from the 15 actinomycetes.

## Conclusion

The results of the present work indicated that the isolates of actinomycetes possess antibacterial and antifungal properties underlining the importance of the actinomycetes in the discovery of new bioactive compounds. We found significant antibacterial and antifungal activity in the ethyl acetate extract of isolates ERI-1 and ERI-3. Methanol extract of ERI-26 exhibited good antibacterial and antifungal activity.

## Conflict of Interests

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

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*Full Length Research Paper*

# cDNA cloning, characterization and expression of cytochrome P450 family 1 (CYP1A) from Javanese medaka, *Oryzias javanicus* by environmental conditions

**Trieu Tuan[1]*, Yoshino Kaminishi[1], Aki Funahashi[1], El-Kady A.H. Mohamed[3], Abeer A. I. Hassanin[2] and Takao Itakura[1]**

[1]Laboratory of Marine Biotechnology, Faculty of Fisheries, Kagoshima University, 4-5-20 Shimoarata, Kagoshima 890-0056, Japan.
[2]Department of Animal Wealth Development, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt. National Institute of Oceanography and Fisheries, Egypt.

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**Cytochrome P450 family 1 (CYP1A) subfamily genes are the most well studied CYP genes in vertebrates; however, information on *CYP1A* genes in Javanese medaka is relatively scarce. In the present study, full-length cDNA of CYP1A was cloned from the fish liver exposed to 500 ppb β-naphthoflavone for 24 h, which is 2439 bp contained in an open reading frame of 1593 bp encoding a protein of 530 amino acids. Real time polymerase chain reaction (RT-PCR) was used to measure the quantitatively tissue expression of the gene by environmental stress conditions. The results indicate that the highest levels of the *CYP1A* gene transcript was in intestine and the lowest in liver of the fish that fed on fuel oil-contaminated feed. Javanese medaka CYP1A transcripts were detected in the gill, muscle and intestine when transferred from seawater to freshwater with the highest level of expression in gill and muscle. CYP1A gene expression in the tissues tends to be down-regulated in Javanese medaka starved for one week.**

**Key words:** Cytochrome P450, CYP1A, Javanese medaka, heavy fuel oil, salinity, starvation, cloning, expression.

## INTRODUCTION

Cytochrome P450 is widely used as an indicator of exposure to environmental contaminants (Hahn et al., 1998). CYP proteins play a critical role in the oxidative metabolism of endogenous compounds, and xenobiotic (exogenous) compounds, including pharmaceuticals and environmental toxins (Diotel et al., 2010). The cytochrome CYP1 family consists of four known subfamilies of vertebrate species (CYP1A, CYP1B, CYP1C and CYP1D), but only CYP1A and CYP1B enzymes appear to be present in all vertebrates and have been studied

*Corresponding author. E-mail: tttuanbl@gmail.com. Fax: +81-99-286-4220.

the most. Mammalian species contain only the CYP1A and CYP1B subfamilies, while the CYP1C subfamily has been detected only in non-mammalian vertebrates (Goldstone et al., 2007). Mammals have two *CYP1A* genes (CYP1A1 and CYP1A2), while most fish have a single *CYP1A* gene (Goldstone et al., 2007).

Javanese medaka (*Oryzias javanicus)* is one of more than 14 species belonging to the genus *Oryzias* distributed in the estuarine regions of East and Southeast Asia (Iwamatsu, 1998; Kinoshita et al., 2009). Medaka has been used as an experimental test fish because it has several advantages, including its ability to adapt to different environmental conditions. Therefore, the fish has been widely used in assessing environmental risk, toxicity testing for new pollutants in various environments, biological response testing and sensitive molecular biomarkers testing (Koyama et al., 2007; Woo et al., 2009)

Baker (1970) and Williams et al. (1994) reports that crude oil and its refined petroleum products contain several toxic organic and inorganic components, which constitutes a significant health risk for both human and other organisms. The ingestion of petroleum hydrocarbon has been reported to induce oxidative stress (Val and Almeida-Val, 1999) by generating free radicals (Achuba and Osakwe, 2003) which leads to lipid peroxidation (Halliwell, 1994) that damages critical cellular macromolecules like DNA, lipids and proteins (Breimer, 1990; Romero et al., 1998; Souza et al., 1999). Petroleum also cause an increased prevalence of morphological abnormalities and reductions in growth and recruitment causing blue sac disease (BSD) in fish, with symptoms of edema, hemorrhaging, deformities and induced CYP1A enzyme (Marty et al., 1997).

Salt stress is an aquacultural and environmental problem considered worldwide. Euryhaline fish, such as the Javanese medaka, can live in both fresh and seawater. However, there are many changes in gene expression and protein activity observed at least 12 to 18 h after exposure to a hyperosmotic environment, suggesting that these effects be mediated immediately by early gene transcriptional factors (Fiol and Kültz, 2005). Indeed, the involvement of such transcriptional factors displaying rapid changes to gene expression after hyperosomotic stress has recently been described in tilapia (*Oreochromis mossambicus*) transferred from freshwater to seawater (Fiol and Kültz, 2005). Functional genomic studies of fish stress responses, particularly the identification of a core set of stress-related transcripts of CYP1 genes, are crucial for a better understanding of their physiological and toxicological functions.

Starvation is a situation undergone and tolerated by many species of fish in their natural environments in response to several factors. To survive these periods of unfavorable feeding conditions, fish reduce their energy expenditures, derived in a high percentage from protein synthesis, and mobilize their endogenous reserves to obtain the energy required to maintain the vital processes (Miriam and Ana, 2011). Starvation may influence 7-ethoxyresorufin-*O*-deethylase (EROD) activities in fish (Jorgensen et al., 1999). Starvation promotes the mobilization of lipids from adipose tissue and/or liver lipid droplets and, thus, the mobilization of the lipophilic toxicants, which are stored in these tissues (Sancho et al., 1998). The effects of starvation on EROD activities are contradictory; in rainbow trout starved for 6 or 12 weeks, a single intraperitoneal injection of benzo[a]pyrene decreased renal EROD activities after six weeks and hepatic EROD activities after 12 weeks of starvation (Andersson et al., 1985). However, in a study by Vigano (1993), 3 weeks of starvation had no influence on hepatic EROD activities, (Vigano et al., 1993) whereas increased liver EROD activities were observed in starved Arctic charr (*Salvelinus alpinus*) previously exposed to the PCB mixture Aroclor 1260 (Jorgensen et al., 1999).

Earlier studies have demonstrated that β-naphthoflavone (BNF) is able to induce (Zhang et al., 1990) the activities of the hepatic CYP1A, UDP-GT and GST in rainbow trout (*Onchorhynchus mykiss*). CYP1A and phenol UDP-GT were induced by BNF in sea bass (*Dicentrarchus labrax*) (Novi et al., 1998). In dab (*Limanda limanda*), only CYP1A was induced; whereas, phase II activities were unchanged (Lemaire et al., 1996). In gilthead seabream *(Sparus aurata*), a proterandrous hermaphrodite species, a hepatic CYP1A isoform has been demonstrated to be inducible by benzo[*a*]pyrene and BNF (Şen and Arinç, 1997). *Takifugu obscurus* CYP1A showed a positive response to BNF exposure (Kim et al., 2008). BNF is an aryl hydrocarbon receptor agonist. AhR is a ligand activated transcription factor that forms a hetero-dimer with the AhR nuclear translocator (ARNT). The AhR-ARNT hetero-dimer binds to XREs. This binding initiates the transcription of several genes of biotransformation enzymes including CYP1A (Whitlock, 1990). From report, Fish differ from mammals in having not one, but at least two *AhR* genes (Hahn, 2002). However, the induction mechanism of CYP1A in fish is believed to be similar to mammals (Pollenz et al., 2002). BNF-induced expression of CYP1A has been reported in many fish species (Sarasquete and Segner, 2000; Chung-Davidson et al., 2004; Jonsson et al., 2007). The mechanism of CYP1A inducted by BNF and other inducers involves the activation of the AhR, which initiates gene transcription (Whitlock, 1990). Here, we report on cDNA cloning and sequence analysis of one of the dominant isoforms of cytochrome P450 gene *CYP1A* from Javanese medaka exposed to 500 ppb BNF over a period of 24 h. We also studied expression patterns of CYP1A mRNA in different tissues caused by environmental factors.

**Table 1.** Oligonucleotide primers used for CYP1A cloning and real time PCR.

| Primer name | Description | Location | Primer sequences (5'-3') |
|---|---|---|---|
| Dgp_CYP1A 1F | Degenerate PCR | 957-976 | GACTCCCTBATTGAYCACTG |
| Dgp_CYP1A 2R | Degenerate PCR | 1229-1248 | TGCCACTGRTTGATGAAGAC |
| Java_CYP1A_GSP 3F | RACE PCR | 203-227 | ACATCGGCCTGGACCGAAATCCTAC |
| Java_CYP1A_GSP 2R | RACE PCR | 306-331 | CCTTTGTTGAGCAGTGTGGGATTGTG |
| Java_CYP1A 1F | Real-time PCR | 1163-1182 | CATTCACAATCCCACACTGC |
| Java_CYP1A 2R | Real-time PCR | 1269-1288 | ATGGATCCTGCCACAGTTTC |
| Java_actin 1F | β-actin RT PCR | 346-365 | AGGGAGAAGATGACCCAGAT |
| Java_actin 2R | β-actin RT PCR | 447-466 | CAGAGTCCATGACGATACCA |

## MATERIALS AND METHODS

### Treatment of fish

Javanese medaka (*Oryzias javanicus*) was cultured in the aquarium facility at the Marine Biotechnology Laboratory, Faculty of Fisheries, in Kagoshima University. Fish were acclimatized to laboratory conditions for one week before the experiment, during which they were fed twice daily on a fine fish commercial diet. After the acclimatization period, eight adult fish were exposed to 500 ppb β-naphthoflavone for 24 h. Liver was dissected, immediately frozen in liquid nitrogen, and stored at -80°C until further use.

### RNA preparation and cDNA synthesis

Total RNA was extracted from the tissue using ISOGEN reagent (Nippon Gene, Japan), according to the manufacturers' protocol. The concentration of RNA was determined by a spectrophotometer (Gene Spec V, Hitachi, Japan). Total RNA concentration and purity were determined spectrophotometrically as described by Sambrook (2001) (Sambrook and Russell, 2001), and the A260/A280 ratio was between 1.7 and 1.9. Poly (A) $^{+}$ RNA was purified using an Oligotex™ -dT30 <Super> mRNA purification kit (Takara Bio, Japan). First strand cDNA was synthesized by the PrimeScript™ 1$^{st}$ Strand cDNA Synthesis Kit (Takara Bio, Japan).

### cDNA cloning and sequencing of CYP1A

Degenerate inosine-containing primers were designed from highly conserved regions, based on the alignment of CYP1A sequences from other fish species. All the primers are listed in Table 1**.** PCR was performed using an Astec PC320 PCR system (Astec Bio, Japan) and TaKaRa Ex Taq polymerase (Takara Bio, Japan) by the following PCR program; initial denaturation step at 94°C for 2 min 30 s and subsequent 35 cycles of amplification (94°C, 30 s; 50°C, 30 s°; 72°C, 30 s) and extension for 2 min at 72°C. The 5' and 3' ends of the CYP were obtained by rapid amplification of cDNA ends (RACE) using the SMART™ RACE cDNA amplification kit (Clontech Takara Bio, Japan) per the supplier's protocol. Gene-specific primers (GSP) were designed based on the sequence obtained from PCR with degenerate primers (Table 1). For cloning, DNA bands of the expected size were excised from the gel, purified using the illustra GFX DNA and gel band kit (GE Health Care, UK), and sub-cloned using pT7 Blue T vector (Novagen, USA) with the ligation-convenience kit (Nippon Gene, Japan). Ligated DNA was transformed into JM109 *E. coli* cells. Purified plasmids were directly sequenced using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, USA). DNA sequencing data was retrieved and edited by Laser gene sequence analysis software (DNAStar ver 5.2). Sequence homology searches were carried out using the basic local alignment search tool (BLAST) program at http://www.ncbi.nlm.nih.gov/BLAST/, whereas sequence alignment was performed using the clustal W program with MegAlign in DNAStar. DNA sequences and their GeneBank accession numbers that were retrieved from the database were used in the analysis presented in Table 2. Phylogenetic and molecular evolutionary analyses were constructed by web-based software using the neighbor-joining method (Dereeper et al., 2010). MatGAT (James et al., 2003) was used to calculate similarity and identity of deduced amino acid sequences of Javanese medaka CYP1s with other CYP1 family members.

### CYP1A expression in various organs of Javanese medaka

#### *Medaka fish and experimental design*

Javanese medaka fish were acclimatized to laboratory conditions for one week before the experiment during which they were fed twice daily on the medaka commercial diet (Kyorin, Japan), and all fish were fed well at the initiation of the treatments. Natural seawater (33-34 ppt) was used for all the experiments. The water temperature was kept at 23±0.5°C. Water pH value ranged from 7.4 to 7.6. The experimental systems were continuously aerated to ensure that the dissolved oxygen levels were adequately maintained.

#### *Oil-contaminated feed experiment*

Heavy fuel oil (bunker C) was used in the experiment. The oil contents of carbon and sulfur residues were <4% and < 2%, respectively (Koyama and Kakuno 2004). Javanese medaka was fed an oil-contaminated feed at levels of 0% (control) and 1%. Thirty (30) medaka fish were divided into two groups and kept in a 12-L tank. Feed was given *ad libitum* during the experiment cycle. Tissue samples were collected after 24 h.

#### *Salinity shock experiment*

Adult Javanese medaka cultured in seawater were starved for two days prior to freshwater transfer, and the change in salinity was

**Table 2.** Gene Bank accession numbers of the CYP1A cDNAs used

| Specie | Gene name | Accession number |
|---|---|---|
| Tilapia (*Oreochromis niloticus*) | CYP1A | FJ389918 |
| Three-spined stickleback (*Gasterosteus aculeatus*) | CYP1A | HQ202281 |
| Plaice (*Pleuronectes platessa*) | CYP1A1 | X73631 |
| Japanese eel (*Anguila japonica*) | CYP1A | AB015638 |
| Rainbow trout (*Oncorhynchus mykiss*) | CYP1A1 | U62796 |
| | CYP1A2 | U62797 |
| Oyster toadfish (*Opsanus tau*) | CYP1A | U14161 |
| Atlantic tomcod (*Microgadus tomcod*) | CYP1A1 | L41917 |
| European flouder (*Platichthys flesus*) | CYP1A | AJ132353 |
| Zebrafish (*Danio rerio*) | CYP1A | AB078927 |
| Leaping mullet (*Liza saliens*) | CYP1A | AF072899 |
| Atlantic salmon (*Salmo salar*) | CYP1A | AF361643 |
| Japanese medaka (*Oryzias latipes*) | CYP1A | AY297923 |
| Indian medaka (*Oryzias melastigma*) | CYP1A | JQ905051 |

ensured by direct transfer from seawater to either freshwater or seawater (control group). Tap water was dechlorinated, and aerated several days prior to its use in the salinity shock experiment. Fifteen (15) medaka fish per group (control and treated) were kept in a 12-L tank. During the freshwater transfer experiments, fish either kept in seawater or transferred into freshwater was sampled after 24 h.

#### *Starvation experiment*

The fish were acclimated to laboratory conditions for one week prior to the study and all fish were fed well at the initiation of the treatments. Two groups (15 medaka fish per group) of adult Javanese medaka were either starved or fed (control group) for 1 week. Fish were kept in a 12-L tank. Fish in the control group were fed twice daily with medaka commercial pellets (Kyorin, Japan).

#### Reverse transcription, primer design and real-time PCR

Total RNA was isolated from liver, gill, muscle, and intestine using QuickGene RNA Tissue Kit S II (RT-2) (Fujifilm, Japan), according to the manufacturer's instruction. Reverse transcription of mRNA was performed with PrimeScript™ first strand cDNA Synthesis Kit (Takara Bio, Japan), following the supplier's protocol. Gene-specific primers for CYP1A and β-actin, as an internal control gene (accession no. JQ905607), were designed by the web-based software Primer3Plus (Andreas et al., 2007) with a product size between 50 to 150 bp, $T_m$ ranging from 57 to 63°C and all the default parameters (Table 1). Real-time PCR was performed using FastStart Essential DNA Green Master Kit and a LightCycler® Nano system (Roche Applied Science). For each sample, gene expression was analyzed in triplicate with the following protocol: initial holding at 95°C for 10 min, 3-step amplification in 45 cycles (95°C for 10 s, 60°C for 10 s and 72°C for 15 s), holding stage at 95°C for 30 s, and melting at 60°C for 20 s and, 95°C for 20 s. Melt curve analysis was performed at the end of each PCR run to assure that the single product was amplified. The $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen 2001) was used to compare the expression levels of the different CYP1A within a given organ, and to calculate changes in fold-induction in response to experimental conditions, using β-actin as a reference gene.

## RESULTS

### Characterization of CYP1A cDNA from Javanese medaka

The full-length CYP1A cDNA was cloned from Javanese medaka liver exposed to 500 ppb β-naphthoflavone for 24 h. The results showed that the full-length cDNA of CYP1A from Javanese medaka contains 2439 bp with an open reading frame of 1593 bp. The deduced protein sequence has 530 amino acid residues with an estimated molecular weight of 60.43 kDa. A 141 bp 5' untranslated region precedes the start codon, and a long 705 bp 3' untranslated region follows the stop codon. Three putative polyadenylated signal sites (AATAAA) were found in the long 3' untranslated region (Figure 1).

### Similarity and identity analysis of the CYP1A cDNA

Table 3 shows the percentage of similarity and identity of deduced amino acid sequences of Javanese medaka CYP1A with other published CYP1A subfamily sequences. Results indicate that CYP1A shared the highest amino acid identity with Indian medaka (*Oryzias melastigma*) CYP1A (93.4%), followed by 90% with Japanese medaka (*Oryzias latipes*) CYP1A, and 79.1% with Leaping mullet (*Liza saliens*) CYP1A1. A lower overall identity was recorded to Atlantic tomcod (*Microgadus tomcod*) CYP1A1 (64.1%). In terms of similarity, Javanese medaka CYP1A shared the highest amino acid similarity with Indian

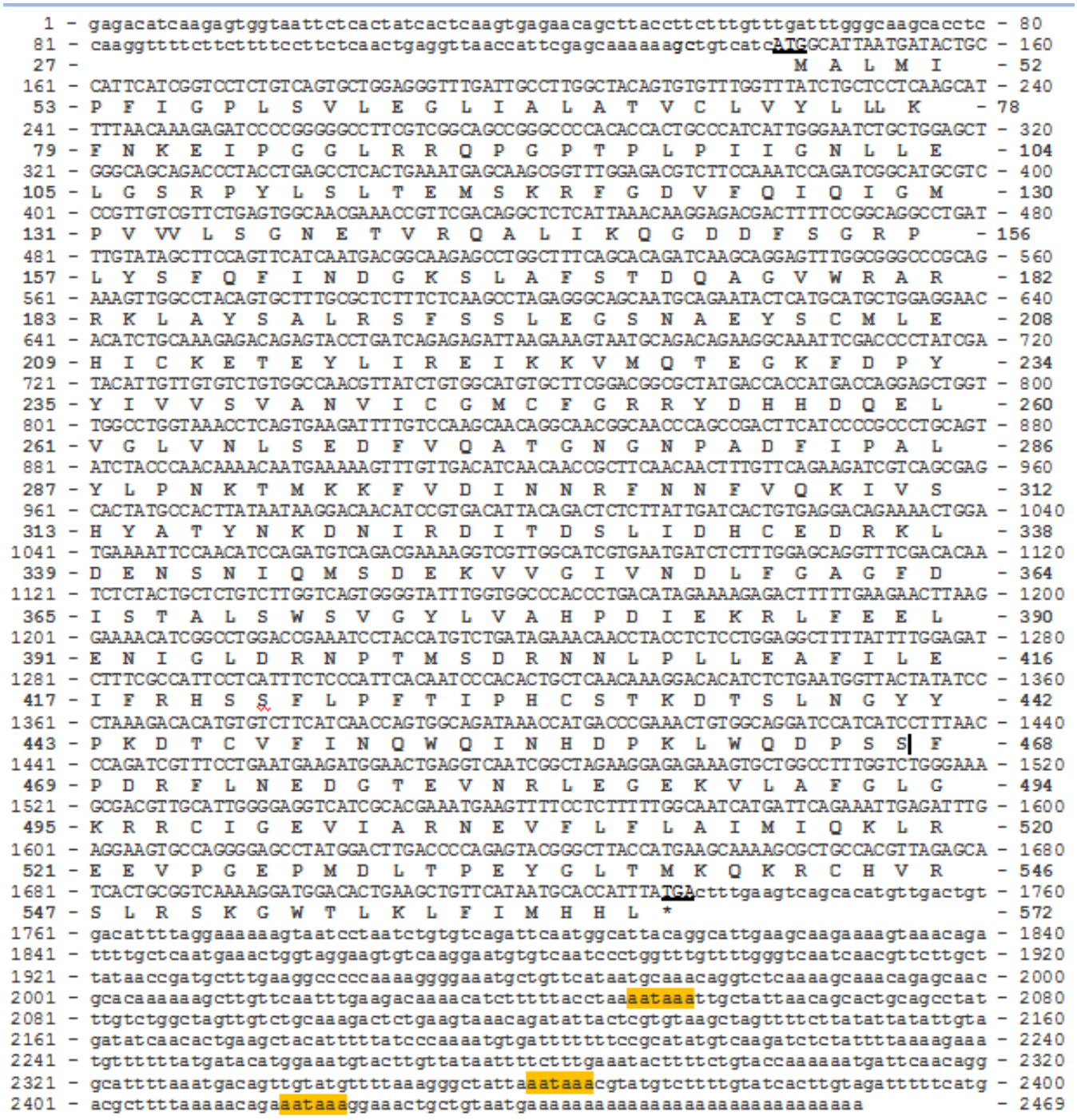


**Figure 1.** Full-length cDNA of CYP1A and the deduced amino acid residues. The coding sequences are shown in uppercase letter whereas lowercase for untranslated regions. The predicted amino acid sequences are in bold letters. The translation start codon and termination codon are underlined and the putative polyadenylation signal (aataaa) is colored in orange.

medaka (*Oryzias melastigma*) (96.6%), followed by Japanese medaka (*Oryzias latipes*) CYP1A (95.8%). The lowest similarity was noted in tomcod CYP1A1 (80.8%).

**Phylogenetic analysis of the CYP1A enzyme**

The phylogenetic tree based on the amino acid

**Table 3.** Percent similarity and identity (upper triangle) of deduced amino acid sequence of Javanese medaka CYP1A with other CYP1A subfamily members

| Parameter | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Java_CYP1A | | 76.8 | 75.5 | 75.7 | 70.9 | 74.7 | 73.6 | 74.3 | 64.1 | 76 | 68.3 | 79.1 | 74.2 | 90 | 93.4 |
| Tila_CYP1A | 88.7 | | 76.8 | 80.2 | 72.7 | 75.3 | 74.7 | 77.4 | 64.8 | 80.6 | 72.4 | 83.3 | 74.7 | 77.9 | 77.9 |
| Stickle_CYP1A | 90 | 91 | | 86.2 | 76.4 | 80.8 | 80.5 | 78.1 | 70.6 | 86 | 72.6 | 82.1 | 80.7 | 75.2 | 76 |
| Plaice_CYPIA1 | 88.5 | 92.3 | 93.5 | | 76.6 | 80.1 | 79.3 | 81.6 | 70 | 97.9 | 73.5 | 85.4 | 80.1 | 77.4 | 77 |
| Jeel_CYP1A | 88.3 | 88.7 | 91.6 | 90.8 | | 80.7 | 79.5 | 74.5 | 68.3 | 76.6 | 74.6 | 77 | 81.2 | 73.1 | 72 |
| Rainbow_CYP1A2 | 89.1 | 89.3 | 91.8 | 91 | 92.3 | | 98.1 | 77.6 | 70.1 | 80.7 | 77.6 | 81 | 97.1 | 75.1 | 74.7 |
| Rainbow_CYP1A1 | 88.3 | 88.7 | 91.4 | 90.2 | 91.8 | 99 | | 77 | 69.2 | 79.9 | 76.8 | 80.3 | 96.6 | 73.9 | 74.3 |
| Toadfish_CYP1A | 88.7 | 91.9 | 91.7 | 93.1 | 90.2 | 90.6 | 89.8 | | 68.2 | 82 | 74.3 | 80.2 | 77.6 | 77 | 76.6 |
| Tomcod_CYP1A1 | 80.8 | 82 | 81.8 | 83.3 | 82.3 | 82.8 | 82 | 82.9 | | 70.6 | 63.1 | 69.1 | 70.7 | 65.2 | 65.5 |
| Flouder_CYP1A | 89.4 | 92.5 | 93.5 | 98.5 | 90.6 | 91 | 90.2 | 92.9 | 83.5 | | 73.1 | 85 | 80.5 | 78.3 | 77.4 |
| Zebra_CYP1A | 84.7 | 87.3 | 88.5 | 89.1 | 89.6 | 90.6 | 90 | 88.7 | 80.5 | 88.9 | | 74.7 | 76.8 | 69.9 | 69.1 |
| Mullet_CYP1A1 | 90.8 | 93.5 | 92.3 | 93.7 | 90.6 | 91.6 | 91 | 93.3 | 85.4 | 93.9 | 89.1 | | 80.7 | 78.9 | 80 |
| Salmon_CYP1A | 88.5 | 89.1 | 91.6 | 91 | 92.1 | 98.9 | 97.7 | 90.2 | 82.8 | 90.4 | 90.6 | 91.4 | | 74.9 | 74.9 |
| *O. latipes*_CYP1A | 95.8 | 90.4 | 91.2 | 89.8 | 89.4 | 89.5 | 88.7 | 90.2 | 82 | 91 | 86.2 | 92.3 | 89.3 | | 93.1 |
| *O. melastigma*_CYP1A | 96.6 | 91 | 91.2 | 89.8 | 89.6 | 90.2 | 89.5 | 90.2 | 82.3 | 90.8 | 86 | 92.3 | 89.5 | 97.9 | |

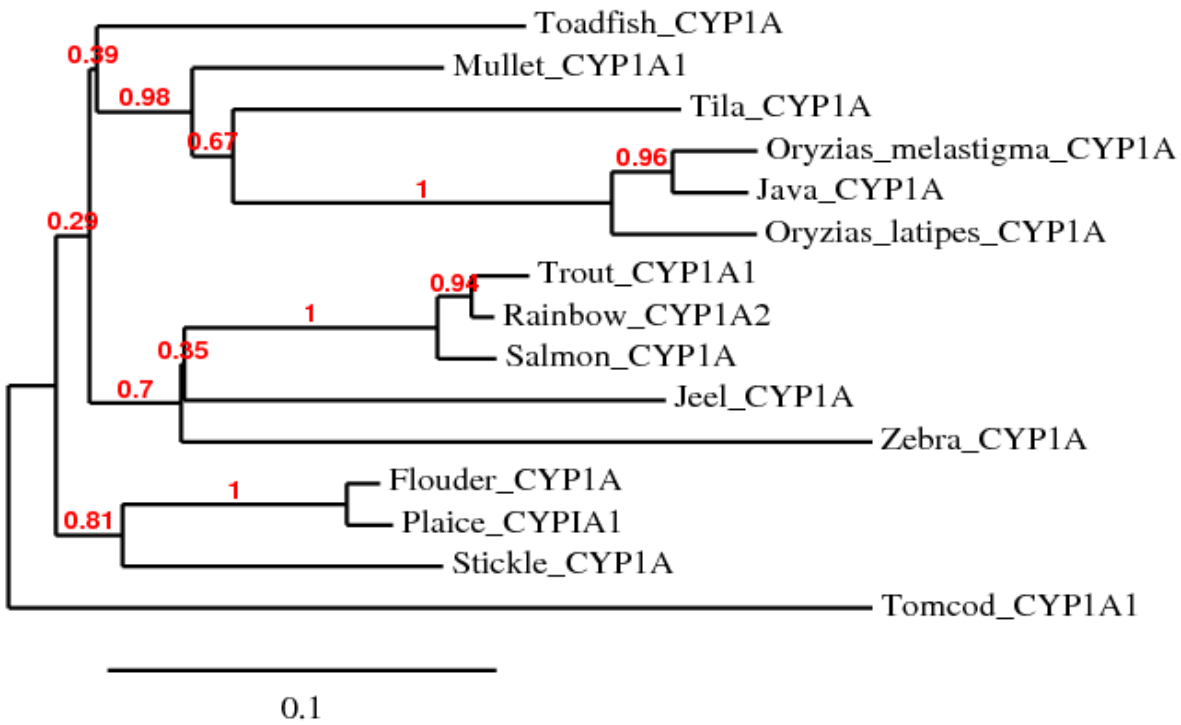


**Figure 2.** Phylogenetic tree of Javanese medaka CYP1A constructed by the neighbor-joining using percent identity of deduced amino acid sequences. The building of the tree also involves a bootstrapping process repeated 100 times to generate a majority consensus tree. *Microgadus tomcod* was used as an outgroup.

sequences, was used to assess the relationship of CYP1A in Javanese medaka with those of other fish species (Figure 2). The phylogenetic tree, with 14 representatives of full-length CYP1A subfamily protein sequences, indicates that the CYP1A clustered with Japanese medaka (*Oryzias latipes*) CYP1A and Indian

medaka (*Oryzias melastigma*) CYP1A1, indicated the evolutionary relatedness.

### Functional domain identification

The deduced amino acid sequences of CYP1A shares a number of characteristics with other cytochrome P450s. Javanese medaka CYP1A N-terminal consists of a proline-glycine rich region as PGPTPLPI. In addition, sequence alignment of Javanese medaka CYP1A enzymes with those of the CYP1A subfamily sequences indicated that Javanese medaka CYP1A contain the five structural conservations around heme-binding core for all cytochrome P450s and six separate substrate recognition sites (SRSs) (Figure 3). The signature motif (FxxGxRxCxG) of the heme-binding core appeared as FGLGKRRCIR. The heme-interacting region of Helix C (WxxxR) was presented as WRARR. The highly conserved residues in Helix I ((A/G) GxxT) showed as GFDTY. The structural conservation played a role in the stabilization of the core structure of cytochrome P450s by hydrogen bond; Helix K (ExxR) was found in the Javanese medaka CYP1A as EIFR. The invariant sequence (PxxFxPE/DRF) proximal to the heme-binding motif is demonstrated as PSSFNPDRF. Figure 3 identifies the six putative substrate recognition sites, (SRS1, SRS2, SRS3, SRS4, SRS5 and SRS6) in the medaka CYP1A gene. The substrate recognition sites (SRSs) of the orthologous Javanese medaka and other species CYP1A subfamily were inferred by the sequence alignment used by (Gotoh, 1992).

### CYP1A mRNA expression in Javanese medaka

### Effect of oil-contaminated feed on CYP1A expression

Real time PCR results showed that the highest expression rates of the CYP1A gene from the Javanese medaka transcript in response to 1% heavy fuel oil-contaminated feed were observed in intestine, and the lowest in liver (intestine>muscle>gill>liver). Although the liver is a major site of expression, not all genes were predominantly expressed in this tissue (Table 4).

### Effect of salinity shock on CYP1A expression

Javanese medaka CYP1A transcripts were detected in most of the tissues examined, including gill, muscle and intestine, but not liver (Table 5). The highest and lowest levels of expression of CYP1A from Javanese medaka were found in gill and liver, respectively (gill > muscle > intestine>liver).

### Effect of starvation on CYP1A expression

Real-time PCR results showed that the CYP1A gene expressed in the tissues analyzed tended to be down regulated in Javanese medaka starved for one week (Table 6). Starvation had no effect on CYP1A induction in all examined tissues, except gill.

## DISCUSSION

Identification of a new CYP1A subfamily expands the diversity of CYP1 genes and presents an opportunity to increase our understanding of the physiological and toxicological significance members of this gene family. In this study, the full-length cDNA, namely CYP1A, were cloned from Javanese medaka. The coding sequences of the identified cDNA were around 1500 bp, which translated into a 500 amino acid protein with a molecular weight around 58 to 60 kDa. Nomenclature of cytochrome P450 is generally based on the identity and evolutionary relationship shared with other cytochrome P450 sequences at the time of discovery, such that genes are grouped in the same family and subfamily when they have > 40% and > 55% amino acid sequence identity, respectively (Nelson 2006). In the present study, Javanese medaka CYP1A was placed in CYP1A family, due to its high identity with most of the CYP1A family members (93.4% with *Oryzias melastigma* CYP1A and 90% with *Oryzias latipes* CYP1A). In addition, molecular phylogenetic analysis shows that Javanese medaka CYP1A and CYP1A subfamily members from other species were grouped in one clade (Figure 2).

The characteristics of the N-terminal region of medaka CYP1A protein exactly match that of the microsomal P450 proteins. At the N-terminal region of medaka CYP1A, a proline-glycine rich region was found, which allows CYPs to function at the ER-membrane. For efficient folding and proper assembly of the P450 proteins, the subsequent proline-glycine rich region acts as a rigid hinge for connecting the membrane anchor and the large catalytic domain, and hence designates the orientation of the catalytic domain in the cytoplasmic side of the ER membrane (Kusano et al. 2001; Kemper 2004). Moreover, several conserved structural elements can be identified from the deduced protein sequence of the Javanese medaka CYP1A gene. The structural conservation around the heme-binding core includes the heme-binding motif (FxxGxRxCxG), helix C (WxxxR), helix I ((A/G)GxxT), helix K (ExxR), and the invariant sequence prior to the heme-binding motif (PxxFxPE/DRF). (Feyereisen 2005) CYP protein has a strongly conserved region surrounding the heme core structure and possesses poorly conserved N- and C-termini regions (Hasemann et al. 1995). Therefore, these structural

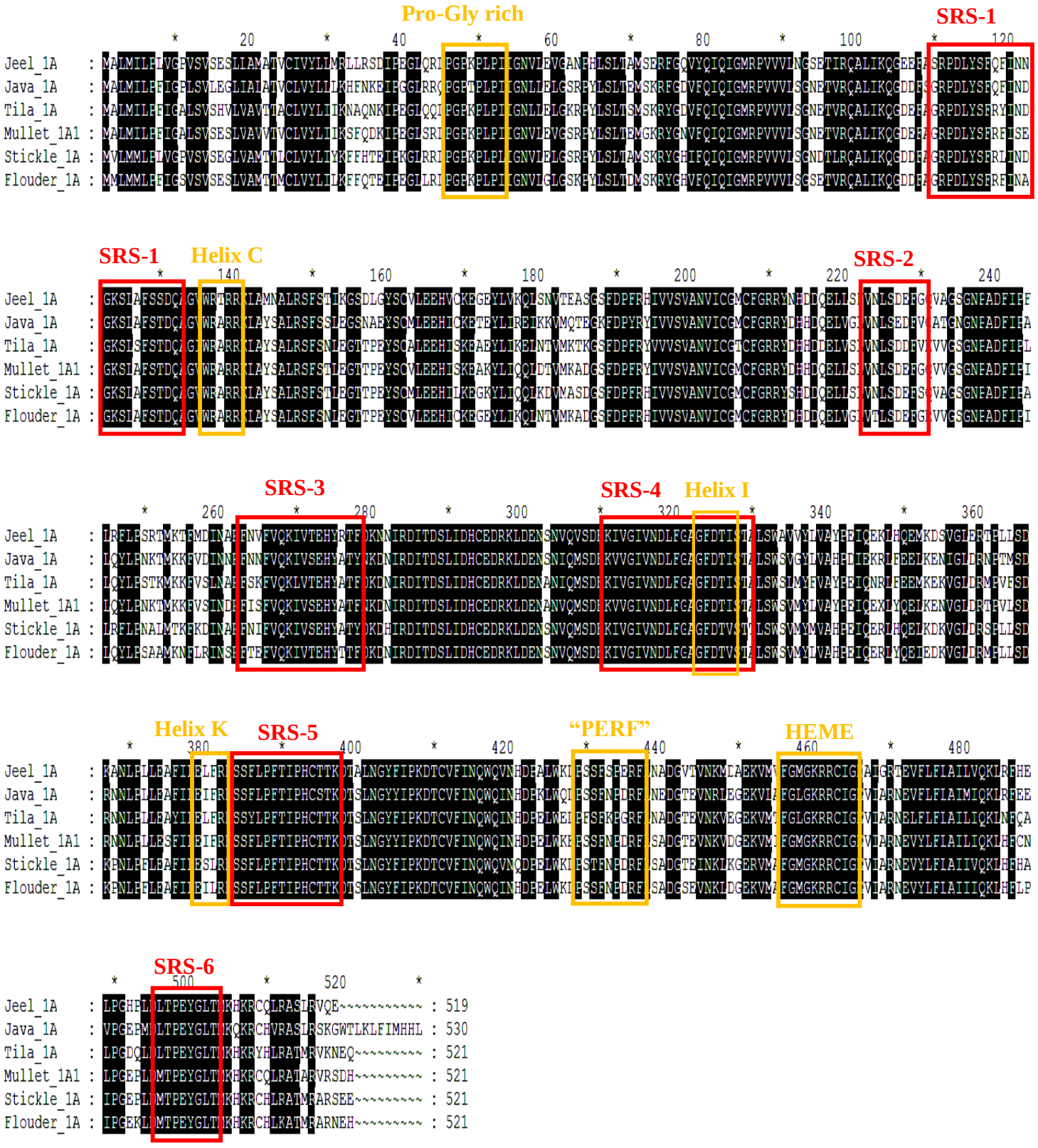


**Figure 3.** Amino acid sequence alignments of Javanese medaka CYP1A with orthologues. Orange boxes indicate the conserved motif region and red boxes indicates substrate recognition sites (SRS).

**Table 4.** CYP1A mRNA expressions in Javanese medaka by oil-contaminated feed

| Sample Name | Oil-contaminated feed | | β-actin | | ΔCt | ΔΔCt | $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|---|---|
| | Mean Ct | Std Dev Ct | Mean Ct | Std Dev Ct | | | |
| L.ctr | 19.62 | 0.12 | 20.34 | 0.15 | -0.72 | 1.74 | 0.30 |
| L.ind | 19.50 | 0.11 | 18.48 | 0.11 | 1.02 | | |
| G.ctr | 23.94 | 0.22 | 18.46 | 0.25 | 5.48 | -2.21 | 4.64 |
| G.ind | 25.65 | 0.19 | 22.38 | 0.25 | 3.27 | | |
| M.ctr | 20.21 | 0.35 | 15.09 | 0.10 | 5.13 | -2.41 | 5.32 |
| M.ind | 16.84 | 0.16 | 14.12 | 0.05 | 2.72 | | |
| I.ctr | 20.94 | 0.13 | 14.40 | 0.11 | 6.54 | -10.28 | 1243.96 |
| I.ind | 24.19 | 0.06 | 27.92 | 0.07 | -3.74 | | |

Amount of CYP1A mRNA, normalized to β-actin mRNA. Relative fold induction was calculated by the equation $2^{-\Delta\Delta Ct}$ using data from carrier control. Data shows ± standard errors of the mean (n=3). L. ctr = liver control; L. ind = liver induced; G. ctr = gill control; G. ind = gill induced; M. ctr = muscle control; M. ind = muscle induced; I. ctr = intestine control; I. ind = intestine induced.

**Table 5.** CYP1A mRNA expressions in Javanese medaka by salinity shock.

| Sample name | Salinity shock | | β-actin | | ΔCt | ΔΔCt | $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|---|---|
| | Mean Ct | Std Dev Ct | Mean Ct | Std Dev Ct | | | |
| L.ctr | 16.75 | 0.14 | 14.61 | 0.13 | 2.14 | 0.68 | 0.62 |
| L.ind | 17.94 | 0.06 | 15.12 | 0.10 | 2.82 | | |
| G.ctr | 21.40 | 0.06 | 16.05 | 0.07 | 5.35 | -4.64 | 24.98 |
| G.ind | 20.76 | 0.08 | 20.05 | 0.05 | 0.70 | | |
| M.ctr | 22.71 | 0.09 | 15.25 | 0.07 | 7.47 | -2.67 | 6.35 |
| M.ind | 25.63 | 0.18 | 20.83 | 0.12 | 4.80 | | |
| I.ctr | 18.87 | 0.14 | 10.68 | 0.12 | 8.19 | -0.80 | 1.74 |
| I.ind | 17.57 | 0.11 | 10.17 | 0.05 | 7.40 | | |

Amount of CYP1A mRNA, normalized to β-actin mRNA. Relative fold induction was calculated by the equation $2^{-\Delta\Delta Ct}$ using data from carrier control. Data shows ± standard errors of the mean (n=3). L. ctr = liver control; L. ind = liver induced; G. ctr = gill control; G. ind = gill induced; M. ctr = muscle control; M. ind = muscle induced; I. ctr = intestine control; I. ind = intestine induced.

consensus sequences have been used as a guideline for cytochrome P450 identification.

In this study, Figure 3 shows the location of the SRS regions within the Javanese medaka CYP1A protein; results shows that SRS1, SRS4, SRS5, SRS6 were highly similar between these CYP1A subfamilies. These SRSs have shown to be conserved among CYP1A orthologous. In contrast, SRS2 and SRS3 exhibit low sequence similarity. These results are similar to the variation in sequence similarities observed between zebrafish CYP1A and CYP1D1 (Goldstone et al., 2009) and in CYP1 genes in killifish (Zanette et al., 2009). The six SRSs may differ in their relative importance among CYPs, but are likely to correspond to regions containing substrate-contacting residues in most CYP genes. These SRS regions have been proposed as crucial for defining substrate specificity for individual CYP isoforms (Gotoh 1992).

The real-time PCR results of this study demonstrate the largest levels of CYP1A mRNA transcript in response to 1% fuel oil-contaminated feed observed in intestine, followed by muscle and gill, respectively. The induction of the CYP1A in intestine may reflect functions associated with the role of the organ in nutrient uptake and processing of body waste products, that is, detoxification of endogenous metabolites, and providing a defensive mechanism against the pollutants entering from the external environment (Hassanin et al., 2009). Chris (2001) studied the specific expression of the CYP1A gene in intestine, gills and liver of tilapia exposed to coastal sediments; there was a large increase in CYP1A transcripts in intestine and liver, respectively (Chris et al., 2001). In addition, Hassanin (2009) reported that benzo[a]pyrene (100 mg/kg injection) induced CYP1A1 mRNA expression in intestine and liver of tilapia, with no detectable expression in the gill and kidney (Hassanin et al., 2009). Similar results were reported by Neilson (2000) and Wong (1996), who found a high level of expression of CYP1A in

**Table 6.** CYP1A mRNA expressions in Javanese medaka by starvation.

| Sample name | Starvation | | β-actin | | ΔCt | ΔΔCt | $2^{-\Delta\Delta Ct}$ |
|---|---|---|---|---|---|---|---|
| | Mean Ct | Std Dev Ct | Mean Ct | Std Dev Ct | | | |
| L.ctr | 26.73 | 0.32 | 28.62 | 0.20 | -1.89 | 5.00 | 0.03 |
| L.ind | 17.30 | 0.19 | 14.19 | 0.09 | 3.12 | | |
| G.ctr | 19.39 | 0.11 | 12.09 | 0.07 | 7.30 | -0.54 | 1.45 |
| G.ind | 20.46 | 0.18 | 13.71 | 0.31 | 6.76 | | |
| M.ctr | 25.35 | 0.18 | 20.69 | 0.09 | 4.66 | 2.79 | 0.14 |
| M.ind | 25.56 | 0.42 | 18.10 | 0.11 | 7.46 | | |
| I.ctr | 16.51 | 0.20 | 9.88 | 0.10 | 6.64 | 1.38 | 0.38 |
| I.ind | 19.83 | 0.10 | 11.81 | 0.40 | 8.02 | | |

Amount of CYP1A mRNA, normalized to β-actin mRNA. Relative fold induction was calculated by the equation $2^{-\Delta\Delta Ct}$ using data from carrier control. Data shows ± standard errors of the mean (n=3). L. ctr = liver control; L. ind = liver induced; G. ctr = gill control; G. ind = gill induced; M. ctr = muscle control; M. ind = muscle induced; I. ctr = intestine control; I. ind = intestine induced.

intestine, with no detectable expression in gills, of tilapia exposed to polycyclic aromatic hydrocarbons (PAHs) (Neilson, 2000). The induction of CYP enzymes in fish liver was first seen as an indicator of aquatic contamination in the 1970s (Zanette et al., 2009). Since then, many studies have shown that CYP genes in vertebrate liver strongly induced by certain organic contaminants that represent a risk to humans and wildlife (Zanette et al., 2009). In this study, no constitutive expression was found in liver. While the lack of induction in the liver was unexpected, it may be because the fish was fed the contaminated food in a short time and thus rapid biotransformation of heavy oil in the liver lowered the concentrations in this tissue.

Many changes to gene expression and protein activity are observed at least 12 to 18 h after exposure to a hyperosmotic environment, suggesting that these effects may be mediated by immediate early gene transcriptional factors (Fiol and Kültz, 2005). In this study, we directly transferred the fish from seawater to freshwater and kept them in the stressful conditions for 24 hours. This result is also consistent with several previous studies. The involvement of such transcriptional factors displaying rapid changes in gene expression after hyperosomotic stress has been described in the gills of tilapia (*Oreochromis mossambicus*) acclimated to seawater from freshwater (Fiol and Kültz, 2005). Fiol (2007) indicated that euryhaline fish can sense and quantify changes in external salinity and activate appropriate compensatory responses (Fiol and Kültz, 2007). After seawater transfer, some parameters are expressed differently with salinity, such as transcription factors, and blood parameters (osmotic pressure, glucose, and cortisol), which the levels of which increased when salinity increased (McGuire et al., 2010). The outcome of the study showed that the expression levels of the biomarker family genes also increased when salinity decreased. Following transfer to freshwater, CYP1A mRNA levels rose, suggesting that this enzyme may play an important role in the salinity stress response developed at the level of the gill and muscle. The present study clearly suggests CYP1A gene involvement in another unexpected physiological function of the Javanese medaka, that is, acclimation to changes in salinity.

Vertebrates differ in their ability to tolerate starvation. Some small birds and mammals may only tolerate one day of starvation, (Mosin 1984; Blem 1990) whereas, some snakes and frogs are reported to survive nearly two years of starvation (de Vosjoli et al., 1995) . Several continuous days of starvation in mammals is a physiological abnormality, whereas fish are generally adapted for extensive periods of starvation. Therefore, the mechanism by which starvation exerts its effects on mammals is probably quite different from that of fish (Andersson et al., 1985; Quabius et al., 2002). The results of the present study showed that CYP1A genes expressed in the tissues analyzed were down-regulated in Javanese medaka starved for 1 week. Several researchers have demonstrated that the induced activity may vary with sex, stage of sexual maturity, food availability, and ambient temperature (Hansson et al., 1980; Forlin et al., 1984; Forlin and Haun 1990; Quabius et al., 2002). However, the present study indicates that the level of induction of the CYP1A gene on Javanese medaka was not influenced by a 1-week period of starvation. Nutritional status (that is, long-term food deprivation) influenced both tissues concentrations and biomarker responses. Food deprivation did not appear to influence hepatic EROD activities or CYP1A content in *Salvelinus alpinus* held for 141 days, either under a restricted feeding regime or without food (Jorgensen et al., 1999). Cytochrome P450-dependent activities towards selected substrates were decreased to varying extents, whereas the liver cytochrome P450 content was not affected by

starvation (Andersson et al., 1985). In this study, the transcription rate of the CYP1A gene was down-regulated by starvation for a 1-week period. This might be a response to the acclimatization strategy in which organisms were fed at certain times each day, so that they prepare themselves for food digestion (Sánchez-Paz et al., 2003).

## Conclusion

In summary, this paper identified and cloned the CYP1A gene in Javanese medaka, an important model species used extensively in environmental toxicology studies. The highest transcript levels in response to 1% heavy fuel oil-contaminated feed were found in the intestine and the lowest in liver. The highest transcript levels were found in gill and muscle when the fish was transferred from seawater to freshwater. The CYP1A gene expressed in the tissues studied was down-regulated in Javanese medaka starved for 1 week. The CYP1A gene from Javanese medaka increased the set of potential biomarkers of environmental stress conditions in fish.

## Conflict of Interests

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

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# Evaluation of essential trace metals in female type 2 diabetes mellitus patients in Nigerian population

**Samuel Oyewole Oyedeji[1*], Adeyemi Adeleke Adesina[2], Olusegun Taiwo Oke[3] and Yetunde Olufunmilayo Tijani[4]**

[1]School of Medical Laboratory Science, Chemical Pathology, Department, Obafemi Awolowo University Teaching Hospital Complex, P.M.B.5538, Ile-Ife, Osun State, Nigeria.
[2]Chemical Pathology Department, Obafemi Awolowo University Teaching Hospital Complex, P.M.B.5538,Ile-Ife, Osun State, Nigeria.
[3]School of Medical Laboratory Science, Haemathology Department, Obafemi Awolowo University Teaching Hospital Complex, P.M.B.5538, Ile-Ife, Osun State, Nigeria.
[4]General Hospital, Ifaki-Ekiti, Ekiti State, Nigeria.

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**Diabetes mellitus (DM) has been reported to be associated with derangement of micronutrients. This study was to investigate the plasma levels of antioxidant elements (zinc, selenium, copper) haemopoeitic elements (chromium and iron) and magnesium in female type 2 diabetes mellitus (DM) patients. Fifty (50) female type 2 DM patients (test subjects) aged 20 to 50 years and 35 healthy, non-diabetic age-matched females (control subjects) were recruited for the study. Fasting blood plasma levels of the elements were determined using atomic absorption spectrophotometer (AAS) after acid digestion. Blood glucose level was determined by glucose oxidase/peroxidase method to confirm the status of the test and the control subjects. Body mass index was determined from weight and height of the subjects. The mean values of plasma zinc, selenium, magnesium and chromium were significantly lower in the diabetic patients than in non-diabetics (P<0.002, P<0.001, P< 0.001 and P<0.001 respectively). The mean values of glucose, BMI, iron and copper was observed to be significantly higher in diabetic patients than control subjects (P<0.002, P<0.05, P<0.002 and P<0.001 respectively). A significant positive correlation existed between glucose and BMI, copper and iron (r=0.717; P<0.0001, r=0.717; P<0.05, r=0.721; P<0.05) whereas an inverse relationship was observed between iron and chromium, selenium and magnesium (r =-0.448, -0.703 and -0.651; p<0.004, 0.000, 0.000 respectively). Negative association was also observed between copper and zinc (r=-0.716; P<0.01). These findings revealed that DM is associated with significant alteration in the concentrations of essential trace metals and significant increase in BMI. This may contribute to various metabolic complications and increased mortality from cardiovascular diseases in DM patients in the rural locality as it as observed among diabetics in urban settings.**

**Key words:** Trace metals, diabetes mellitus, copper, chromium, Ekiti state.

## INTRODUCTION

Diabetes mellitus (DM) represents today a disease with massive spreading with medico-social consequences. Recent research has shown a close relationship between some specific micronutrients and this disease, with impli-

cations for the pathogenesis of this disease and its vascular complications. The relationship between nutrition and diabetes was suspected as early as 1674 and over the last 20 years, numerous studies have found alterations in micronutrients status of patients with diabetes mellitus (Mooradian and Morley, 1987). There are accumulating evidences that the metabolism of several trace metals are altered in diabetes mellitus and these micronutrients might have specific roles in the pathogenesis and progression of the disease (Akinloye et al., 2010). In particular, DM has been shown to be associated with abnormalities in the metabolism of zinc, chromium, copper, magnesium and manganese (Retnam and Bhandarkar, 1981). People with type 2 DM have greater excretion and lower tissue levels of chromium than non-diabetic control (Musad et al., 2004). Evidence that systematic iron overload could contribute to abnormal glucose metabolism was first derived from the observation that the frequency of diabetes is increased in classic hereditary hemochromatosis (HH) (Sundararaman et al., 2007). Zinc is involved in the regulation of insulin receptor-initiated signal transduction mechanism. Zinc deficiency is associated with a number of metabolic disturbances including impaired glucose tolerance, insulin degradation, and reduced pancreatic insulin content (Nsonwu et al., 2006). Copper level has been found to be high in people with diabetes compared to those without. The higher the copper level, the more likely the complications from diabetes (Kumar et al., 2007). Magnesium is an essential cofactor for multiple enzymes involved in glucose metabolism and is hypothesized to play a role in glucose homeostasis, insulin action and in the development of type 2 diabetes (Larsson and Wolk, 2007).

The objective of this study was to investigate the levels of essential trace metals (Mg, Cr), antioxidant related trace metals (Zn, Cu, and Se) and iron (Fe) in type II diabetes mellitus.

## MATERIALS AND METHODS

### Study location

The study was carried out in general hospital, Ikole-Ekiti, Ekiti state, South Western region of Nigeria. The hospital is a secondary health institution provided by the Ekiti state government in Nigeria to serve as a health care providing centre in this town and the adjourning towns and villages. Ikole-Ekiti is a rural town with the majority of the inhabitants and surrounding towns being farmers and traders.

### Study population

The study population was known as female type II DM patients attending the hospital and apparently healthy non-diabetic age-matched individuals in the same locality.

### Study design

The study was a stratified randomized study designed to investigate the status of trace metals in female type 2 DM patients in rural community.

### Exclusion criteria

These included proteinuria, pregnancy, lactating mothers and alcoholism. Patients on prolonged medication that could alter measured metals such as chelating agents, D-penicillamine, or oral contraceptive pills were excluded from the study.

### Subjects population

The subjects were recruited from patients attending general hospital, Ikole-Ekiti, Ekiti State, Nigeria. 110 women who gave their consent after adequate education on purpose of the study were recruited for this study. 85 women participated in the study. The subjects were grouped into two categories comprising of 50 female type 2 DM patients (test subjects) and 35 apparently healthy non-diabetic women tagged control subjects. Questionnaires were administered among the subjects to obtain socio-demographic information.

### Determination of body mass index (BMI)

This was done by measurement of height in metre (m) and weight in kilogramme (Kg) using standard methods. The BMI was calculated from the average height and weight as follow:

$BMI = Weight\ (kg)/Height^2\ (m^2)$.

### Blood sample collection

After an overnight fast of 10 to 12 h, venous blood was collected from the cubital vein of the participants using sterile hypodermic syringe into lithium heparin sample bottles for trace metals estimation, and fluoride oxalate bottles for fasting blood glucose. The sample in lithium heparin bottles were centrifuged, the plasma was separated and stored frozen at -20°C till analysis was carried out.

### Biochemical analysis

Blood glucose was determined using standard enzymatic spectrophotometric method.

### Trace metals determination

Concentrations of the trace metals were estimated using flame atomic absorption spectrophotometry after deproteinization of the plasma by adding 2 ml of the sample diluted to 10 ml with dilute hydrochloric acid. The diluted sample was then centrifuged at 3000 revolution /min for 30 seconds and the supernatant aspirated directly into the flame. The principle is based on dissociation of the element (by the flame) from its chemical bonds. This is then placed

*Corresponding author. E-mail: oyedejis1214@yahoo.com. Tel: +2348033822818.

**Table 1.** Characteristics of parameters in test and control subjects (Mean±SEM) and comparison of mean using students' t-test.

| Parameter | Test group (N=50) Mean ± SEM | Control group (N=35) Mean ± SEM | T-test | P-value |
|---|---|---|---|---|
| Zinc (µmol/L) | 18.15 ± 0.33 | 21.86 ± 0.06 | 40.607 | P < 0.002 |
| Selenium (µmol/L) | 0.47 ± 0.03 | 1.02 ± 0.01 | 127.75 | P < 0.001 |
| Iron (µmol/L) | 28.83 ± 0.45 | 22.27 ± 0.02 | 68.121 | P < 0.002 |
| Magnesium (µmol/L) | 0.55 ± 0.01 | 1.13 ± 0.02 | 109.78 | P < 0.001 |
| Copper (µmol/L) | 1.81 ± 0.02 | 0.61± 0.30 | 112.00 | P < 0.001 |
| Chromium (µmol/L) | 0.96±0.08 | 5.39 ± 0.84 | 132.80 | P < 0.001 |
| Glucose (mmol/L) | 13.41 ± 0.66 | 4.34 ± 0.20 | 61.33 | P < 0.002 |
| BMI (kg/m²) | 25.52 ± 0.49 | 21.66 ± 0.81 | 15.89 | P < 0.05 |

BMI-Basal metabolic rate.

in unexcited or ground state (neutral atom). The neutral atom at low energy level is capable of absorbing radiation at a very narrow bandwidth corresponding to its own line spectrum (Kaneka, 1990).

#### Statistical analysis

The data obtained were processed statistically using statistical package for social sciences (SPSS Inc., Chicago, IL software, version 14.3). Differences were significant when the p-value was <0.05. Data obtained from the study were expressed as means and standard error of mean. The differences in the means were compared using student t-test and Pearson's correlation was used to determine the association between variables.

## RESULTS

Table 1 shows the mean ± standard error of mean of all the parameters estimated and the result of student t-test comparing mean of the test and the control subjects. The mean plasma fasting blood glucose and BMI of test subjects were significantly higher than that of the control subjects (P<0.002 and P<0.05 respectively). The plasma concentrations of zinc, selenium, magnesium and chromium were all significantly lower in test subjects than control subjects. (P<0.002, P<0.001,P<0.001, P<0.001, and P<0.001 respectively). However, the mean plasma concentrations of iron and copper in diabetic patients were found to be significantly higher (P<0.002) than that in control subjects. Table 2 shows the result of Pearson's correlation of parameters with one another. There was strong positive relationship between fasting blood glucose and iron (r=0.721, P<0.001), copper (r=0.815, P<0.001) and BMI (r=0.717, P<0.001). Negative correlation was observed between fasting blood glucose and zinc (r= -0.479, P<0.000), selenium (r= -0.669, P<0.001), magnesium r=-0.584, P<0.001). Zinc, selenium, magnesium were positively correlated with one another. But they had inverse relationship with iron.

## DISCUSSION

This study showed that there was derangement in the levels of trace metals and increased BMI in type 2 diabetes mellitus (DM). Some trace metals act as antioxidants preventing the deleterious activities of oxidants on membranes while others act directly as co-factors in metabolism of macro molecules such as glucose. Diabetes has been associated with abnormalities in the metabolism of these trace metals (Schlienger et al., 1988). In this study, zinc level was found to be significantly lower in DM patients (P< 0.002) than in healthy individuals. Zinc has been found to have insulin-like effects in that it enhances glucose up take by inhibiting glycogen synthesis (Diwan et al., 2006). The findings of past studies on zinc level in type 2 DM has been mixed. Some have reported low Zinc levels in diabetes (Nakamura et al., 1991), some have reported higher level (Zargar et al., 2002) and some others reported no significant difference (Babalola et al., 2007). The present study corresponds with those that have reported lower levels. Selenium is known to act as antioxidant and peroxynitrite scavenger when incorporated into selenoprotein (Beytht and Akasakai, 2003). It is the main element in glutathione peroxidase which acts as an active antioxidant enzyme that reduces formation of free radicals and peroxides of lipoproteins. Selenium level was found to be significantly lower in diabetic than in non-diabetic group in this study. This finding corresponds with the report of Burt (2007); Akinloye et al. (2010). Selenium has strong positive correlation with zinc and magnesium in this study. Iron is an essential element for wide varieties of metabolic processes but it also has the potential to cause deleterious effects by formation of toxic oxygen radicals that can attack all biological molecules (Halliwel and Gutteridge, 1999). There are suggestive evidences that iron plays pathogenic roles in diabetes mellitus and its complication such as micro angiopathy and arteriosclerosis (Swaminathan et al., 2007). The concentration of plasma iron of diabetics in this study was found to be significantly higher than in control subjects (P< 0.002) and has strong positive correlation with copper, glucose and BMI. Our finding suggests that one of the causes of oxidative stress in type 2 diabetes mellitus, this may be

**Table 2.** Pearson correlation showing level of association in the BMI and biochemical parameters among female type 2 DM patients.

| Parameter | Zn | Se | Fe | Mg | Cu | Glucose | BMI |
|---|---|---|---|---|---|---|---|
| Zinc | 1 | 0.635** | -0.450* | 0.558** | -0.717** | -0.479** | -0.331** |
| Selenium | 0.635* | 1 | -0.703* | 0.544** | -0.844** | -0.669** | -0.440* |
| Iron | -0.430* | -0.703** | 1 | -0.651** | 0.811** | 0.721** | 0.458* |
| Magnesium | 0.558* | 0.544** | -0.651** | 1 | -0.698** | -0.584** | -0.427* |
| Copper | -0.716* | -0.884** | 0.811* | -0.898** | 1 | 0.815** | 0.581* |
| Glucose | -0.479* | -0.669** | 0.721* | -0.584** | 0.815** | 1 | 0.717* |
| BMI | -0.333* | -0.440** | 0.458* | -0.427** | 0.581** | 0.717** | 1 |

** Correlation is significant at the 0.01 level. * Correlation is significant at the 0.05 level. BMI = Basal metabolic rate.

the accumulation of plasma iron and copper which can be involve in Fenton reaction.

This study shows a significantly lower concentration ($P<0.001$) of magnesium level in the test subjects when compared with the control subjects. This finding correlates with the earlier studies (Purvis and Movahed, 1992). Magnesium is needed for the synthesis and secretion of insulin, it also enhance cells uptake of insulin and therefore keep up their insulin sensitivity. The reduced insulin levels in diabetes leads to hyperglycemia (Jerry and Nadler, 2000). Hypermagnesuria in diabetics have been attributed to osmotic diuresis. Glycosuria, which accompanies diabetic state, impairs renal tubular reabsorption of magnesium from the glomerular filtrate (Garland, 1992) and likely contributes to high frequency of hypomagnesaemia in poorly controlled diabetics (Nsonwu et al., 2006). Copper is an important trace metal for various metabolic enzymes. Its deficiency has been attributed to anemia and low level HDL-cholesterol (Sreedhar et al., 2004). Serum copper concentration in this study was found to be significantly higher in DM patients ($P<0.001$) than in non diabetes. The finding correlates with that of Walter and his colleagues (1991) but at variance with that of Babalola et al. (2007). Diabetes has been shown to be associated with abnormalities in the metabolism of chromium and its impairment has been reported as aggregating factor in the progression of the disease. It is a cofactor in the action of insulin so it controls the action of insulin (Kimura, 1996). In this study, there was significantly higher concentration of chromium in non diabetic individuals than that in the diabetes patients ($P < 0.001$). This report corresponds to the finding of Morris et al. (1999). The body mass index (BMI) of the diabetics was found to be significantly higher ($P<0.05$) than that of the non diabetes and has strong positive correlation with iron, copper, and glucose. Increased BMI has been shown to be associated with type 2 DM. The observation in this study correlates with that of Anjum and Arbab (2010). The study established that DM patients are often overweight (with BMI >25) and this is independent of the socioeconomic status.

In conclusion, this study demonstrate significant reduction of the essential trace (zinc, selenium and magnesium) metals in type 2 DM patients and significant increase in copper, chromium, iron, glucose and BMI. These alterations may be responsible for different metabolic disturbances, oxidative stress and other complications associated with diabetes mellitus.

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# Investigation of carrier oil stabilized iron oxide nanoparticles and its antibacterial activity

**K. L. Palanisamy[1]*, V. Devabharathi[2] and N. Meenakshi Sundaram[3]**

[1]Department of Physics, Sengunthar Engineering College, Tiruchengode, India
[2]Department of Physics, KSR Institute of Engineering and Technology, Tiruchengode, India
[3]Department of Biomedical Engineering, PSG College of Technology, Coimbatore, India

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**Iron oxide nanoparticles were synthesized by co-precipitation method. The polyunsaturated carrier oil (flaxseed oil) is used as a stabilizing agent for iron oxide nanoparticles. Kirby Bauer method was used to investigate the antibiotic sensitivity of carrier oil stabilized and uncoated SPIONs at 10 and 20 µg/L on Gram-positive bacterium *Bacillus cereus* (vegetative cell). The nanoparticles were characterized by X-ray diffraction method (XRD), Fourier transform infrared red (FTIR) analysis, particle size analyzer and Transmission Electron Microscopy. Structure of magnetite nanoparticles was confirmed by XRD analysis and the estimation of nanoparticle size was confirmed with TEM. The attachment of functional groups of oil was predicted using FTIR spectroscopy. This comparison study revealed that carrier oils stabilized iron oxide nanoparticles show more antibacterial activity than the bare iron oxide nanoparticles.**

**Key words:** Iron oxide nanoparticles, flaxseed oil, *Bacillus cereus*

## INTRODUCTION

The Iron oxide nanoparticles can be synthesized by co-precipitation method. The nanoparticles of iron oxide such as $Fe_3O_4$ and γ-$Fe_2O_3$ are very prominent materials in biomedical applications (Sun et al., 2007; Lee et al., 2004; Pan and Yu, 2009). The prevention of agglomeration is critical a factor during the synthesis and it can be controlled by appropriate stabilizer, but the stabilizing agent should ensure its function and not to have any effect on the toxicity of the nanoparticles. In this work, flaxseed oil (linseed oil) is used as a stabilizing agent. Flaxseed oil is a colourless to yellowish oil obtained from the dried, ripened seeds of the flax plant (*Linum usitatissimum*, Linaceae). Flax-based oils are sought after as food because of their high levels of poly unsaturated α-linolenic acid (C18:3) (Figure 1), which is a one form of omega-3 fatty acid. The other fatty acids, oleic acid (monosaturated - omega -6 - C18:1), saturated palmitic acid (C16:0) and stearic acid (C18:0) are also constituted (Angerer and von Schacky, 2000; Balk et. al., 2006; Barceló-Coblijn et al., 2008; Barre et al., 2009). Linseed oil is an edible oil marketed as a nutritional supplement (Bassett and Rodriguez-Leyva, 2009). Some

*Corresponding author. E-mail: klpphysics@gmail.com. Tel: +9199651-39339. Fax: 91-4288-255716.

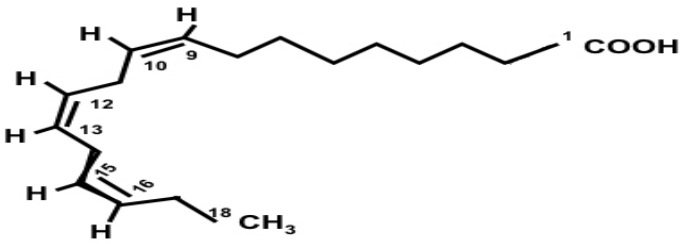


**Figure 1.** Structure of α-linolenic acid.

studies suggest that α -linolenic acid (a substance found in flaxseed and flaxseed oil) may benefit people with heart disease (Bays 2007; Hou et al., 2007; Ye et al., 2006).

The antibacterial test on *Bacillus cereus (B. cereus*) was studied using this flaxseed oil stabilized iron oxide nanoparticles. *B. cereus* is a bacterium that is commonly associated with large outbreaks of food borne illness. *B. cereus* has a wide distribution in nature, frequently isolated from soil and growing plants, but it is also well adapted for growth in the intestinal tract of insects and mammals.

## MATERIALS AND METHODS

All the chemicals were of analytical reagent graded and used without further purification. Ferrous sulphate ($FeSO_4$, 99%), ferric chloride ($FeCl_3$, 99%) and Sodium hydroxide (NaOH) were obtained from Merck (India). Flaxseed oil was obtained from Falcon Industries, India. The Gram-positive bacterium *B. cereus* was purchased from Institute of Microbial Technology (Chandigarh, India), India.

Coprecipitation method was adopted for making the iron oxide nanoparticles. A 100 ml of 0.4 mol/L solution $FeCl_3$ and 100 ml of 0.2 mol/L $FeSO_4$ were mixed and dissolved in deionized water. Then 2 mol/L of sodium hydroxide was added into the above solution and the pH value between 10 to 11 was maintained with continuous stirring using a magnetic stirrer for 1 h and a dark precipitation was formed. 5 ml of flaxseed oil was taken and heated to 80°C in hot air oven and added in the precipitated solution for stabilization. The precipitated iron oxide particles were washed several times with double distilled water and filtered. Then it was dried at 150°C for 2 h and grinded to fine powder. Then the same procedure was followed for preparing bare iron oxide nanoparticles without adding of flaxseed oil.

X-Ray diffraction (XRD) patterns were recorded with a Philips analytical X-ray diffractometer using CuKα radiation (λ= 1.5406 Å). FTIR spectra were performed and recorded with a Fourier transform infrared spectrophotometer of type Nicolet 870. TEM was recorded using Philips CM12 model. Particle size analysis was done by Malvern (U.K.) Make 2000E model.

### Determination of antibacterial activity by well-diffusion method

Antimicrobial assay for the synthesized iron oxide nanoparticles were performed against Gram-positive *B. cereus* by Kirby-Bauer disk diffusion method. The pure cultures of organisms were sub-cultured in Müller-Hinton broth at 121°C at 15 psi for 45 min in an autoclave. The medium is poured into sterile Petri plate and incibated at 37°C for 24 h to check the plate sterility. The overnight grown *B. cereus* ($4\times10^9$) count was taken and swabbed on three dimensional lawn types on Mueller Hinton agar (MHA) plates. The sterile disc was coated with 10 µg/L of each nanoparticle fix on the top surface of the medium. In another case 20 µg/L of each nanoparticle was coated and fixed on the top surface of the medium. The plates were incubated at 37°C for 24 h and observed for every 4 h. It was observed that the zone of lysis was increasing on prolonged incubation. After 24 h incubation the plates were examined for the appearance of zone of inhibition. The zone of inhibition was measured in mm and recorded.

## RESULTS AND DISCUSSION

XRD analyses confirmed that the synthesized nanoparticles were iron oxide nanoparticles as shown in Figure 2. The characteristic peaks were marked by their 2θ angles and compared with JCPDS data. The planes (2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1) and (4 4 0) were observed for the corresponding angles of both the samples. The particle sizes were evaluated from the XRD data using the Debye - Scherrer equation and the average particle sizes of the flaxseed oil stabilized iron oxide nanoparticles were 32 and 52 nm for the uncoated sample.

The surface modification of the iron oxide nanoparticles with their stabilization was confirmed by Fourier transform infrared spectroscopy measurements (FTIR) (Figure 3). The presence of absorption peaks in the region of wave numbers 550 to 630 $cm^{-1}$ corresponding to the Fe-O vibration. The peaks at 3434 $cm^{-1}$ in Figure 2a and 3442 $cm^{-1}$ in Figure 2b were related to the vibrations of ¯OH and the peaks at 1707, 1629, 1706 and 1631 $cm^{-1}$ were due to the overlapping of the absorption bands of the carboxyl groups and double bond of oleic acid and α-linolenic acid, respectively (Lu et al., 2012). The other peaks observed in the region of 882 to 1366 $cm^{-1}$ in sample (b) were due to the additional compounds (polyphenols, peroxides and polycyclic aromatic hydrocarbons (PAHs), vitamin K and E) present in the oil.

The particle size and the morphology of iron oxide nanoparticles were observed by transmission electron microscopy (TEM). Figure 4 shows TEM micrographs of samples of uncoated and flaxseed oil stabilized iron oxide nanoparticles and these images indicate that the particles are not aggregated and the mean physical sizes were obtained by Debye-Scherer formula. The antibacterial activity of iron oxide nanoparticles of uncoated (a) and flaxseed oil mediated samples were performed against Gram- positive bacterium *B. cereus* (vegetative cell) at 10 µg/L and 10 µg/L concentrations using agar well diffusion method. The values of zone of inhibition (mm) of these nanoparticles were presented in the Table 1.

Figures 5 and 6 show the zone of inhibition of bacterial growth on agar plates with two different concentrations of flaxseed oil stabilized and bare iron oxide nanoparticles. It was observed from the images and pie chart that the growth of bacteria was inhibited gradually with increase in

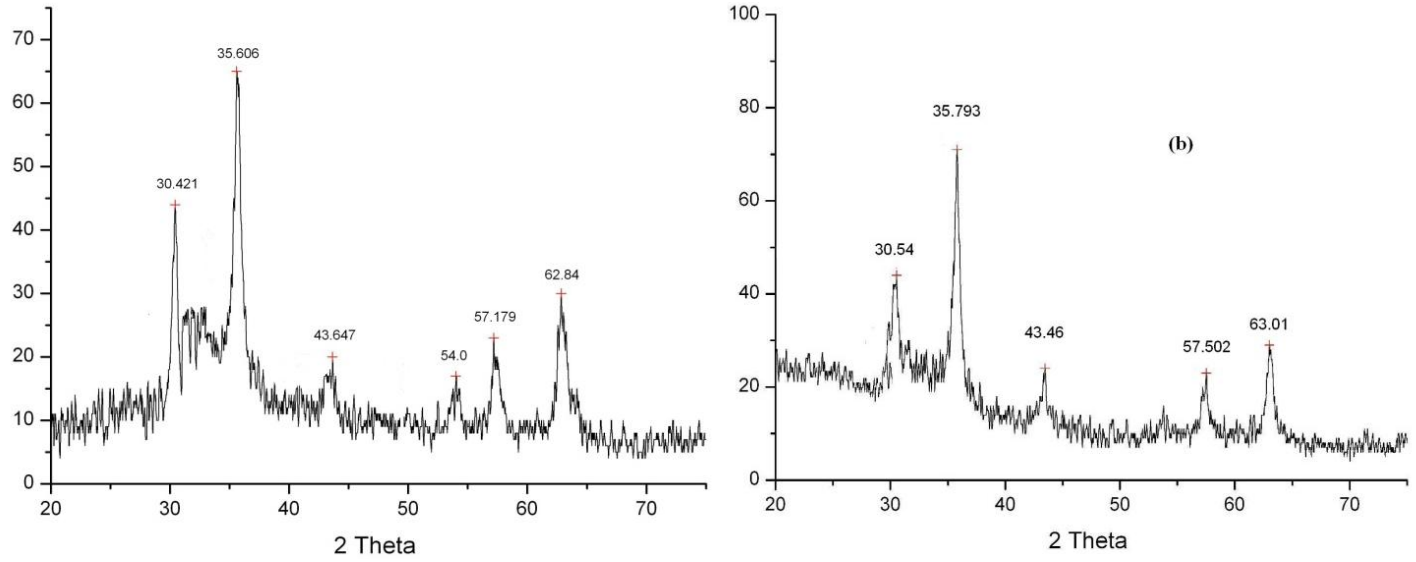


**Figure 2.** XRD patterns of uncoated (a) and flaxseed (b) iron oxide nanparticles.

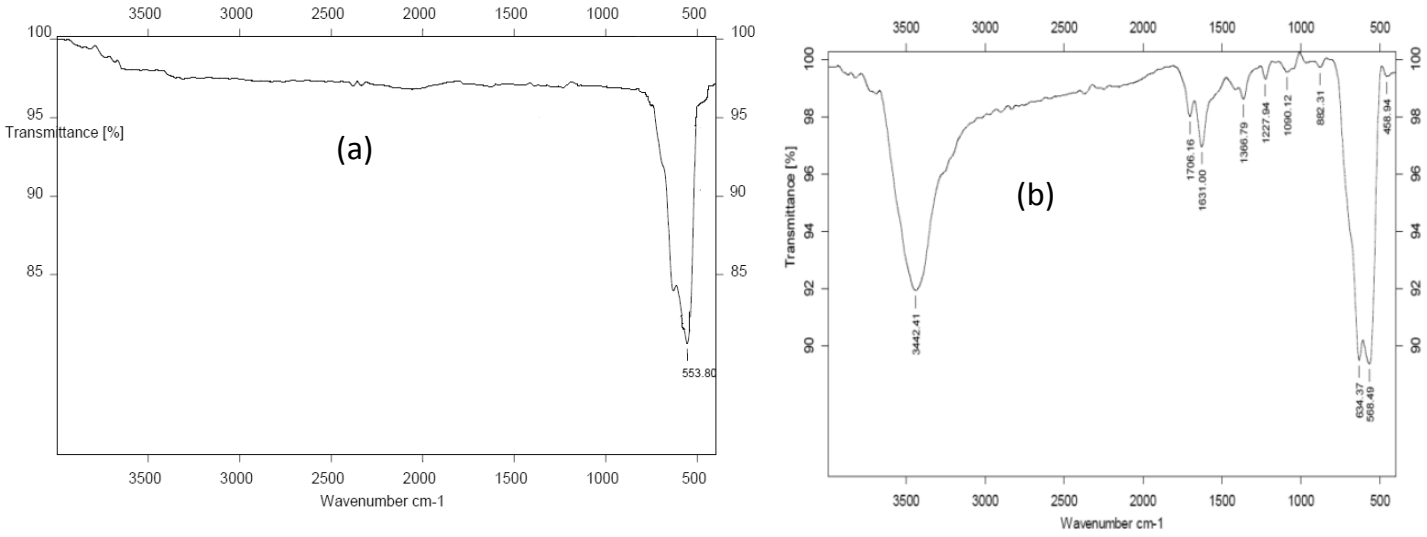


**Figure 3.** FTIR analysis of uncoated (a) and flaxseed (b) iron oxide nanoparticles.

concentration of iron oxide nanoparticles. Further the results clearly demonstrate that Flaxseed oil mediated iron oxide nanoparticles could promise a better antimicrobial agent than the bare iron oxide nanoparticles.

## Conclusion

In the field of nanotechnology, the development of reliable and eco-friendly processes for synthesizing of metal oxide nanoparticles is very essential. Here, we have reported a simple, eco-friendly and low-cost approach for preparation of magnetite nanoparticles by reduction of ferric chloride solution with a green method using flaxseed oil as the stabilizing agent and compared with the uncoated iron oxide nanoparticles. The characteristics of the obtained iron oxide nanoparticles were studied using FTIR, XRD and TEM techniques. The antibacterial effects of flaxseed oil stabilized iron oxide nanoparticles and bare iron oxide nanoparticles against gram- positive bacterium bacillus cereus (vegetative cell) were investigated.

The results exhibit that the flaxseed oil stabilized iron oxide nanoparticles were less in size, more antibacterial activity than the bare iron oxide nanoparticles.

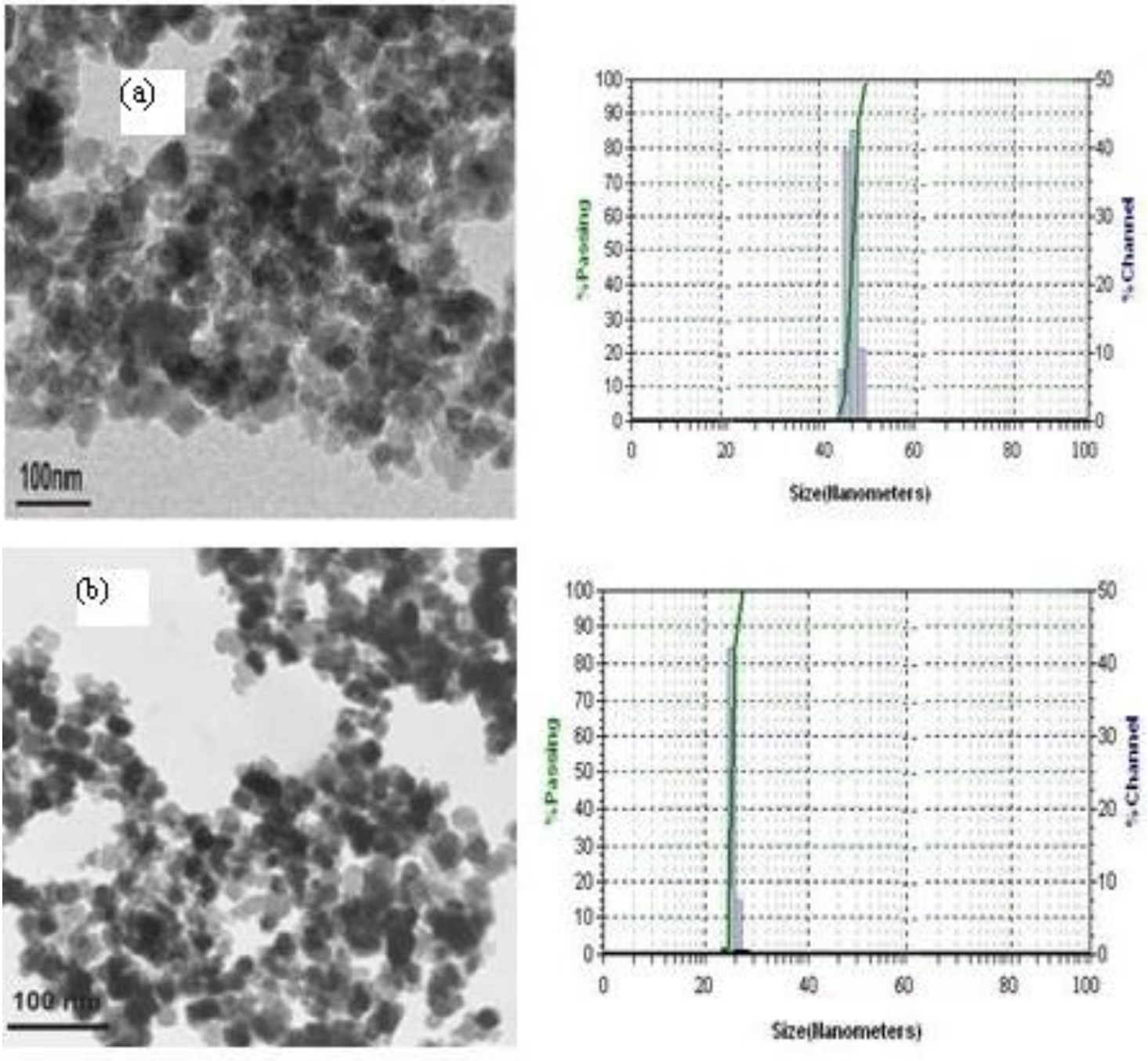


**Figure 4.** TEM photographs of uncoated (a) and flaxseed (b) SPIONs.

**Table 1.** Zone of inhibition (mm) for various concentrations of ncoated (a) and flaxseed (b) SPIONs.

| 10 µg/L of nanoparticle coated | | | 20 µg/L of nanoparticle coated | | |
|---|---|---|---|---|---|
| **S/N** | **Nanoparticle** | **Zone of inhibition (mm)** | **S/N** | **Nanoparticle** | **Zone of inhibition (mm)** |
| 1 | Flaxseed oil stabilized SPIONs | 3.75 | 1 | Flaxseed oil stabilized SPIONs | 8.5 |
| 2 | Uncoated SPIONs | 1.5 | 2 | Uncoated SPIONs | 3.5 |

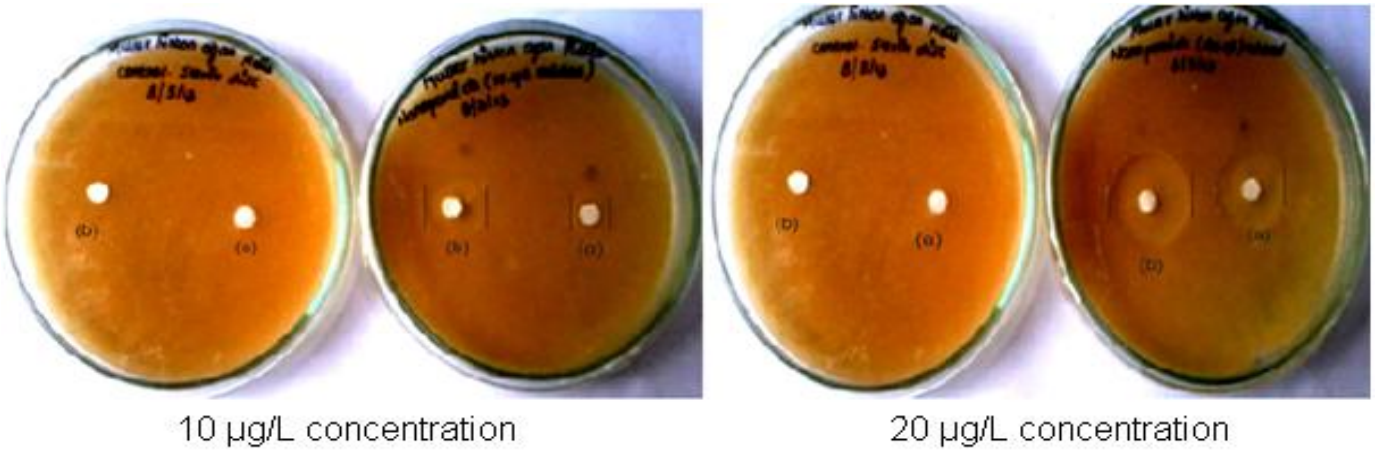


**Figure 5.** Zone of inhibition (mm) of uncoated (a) and flaxseed (b) iron oxide nanoparticles

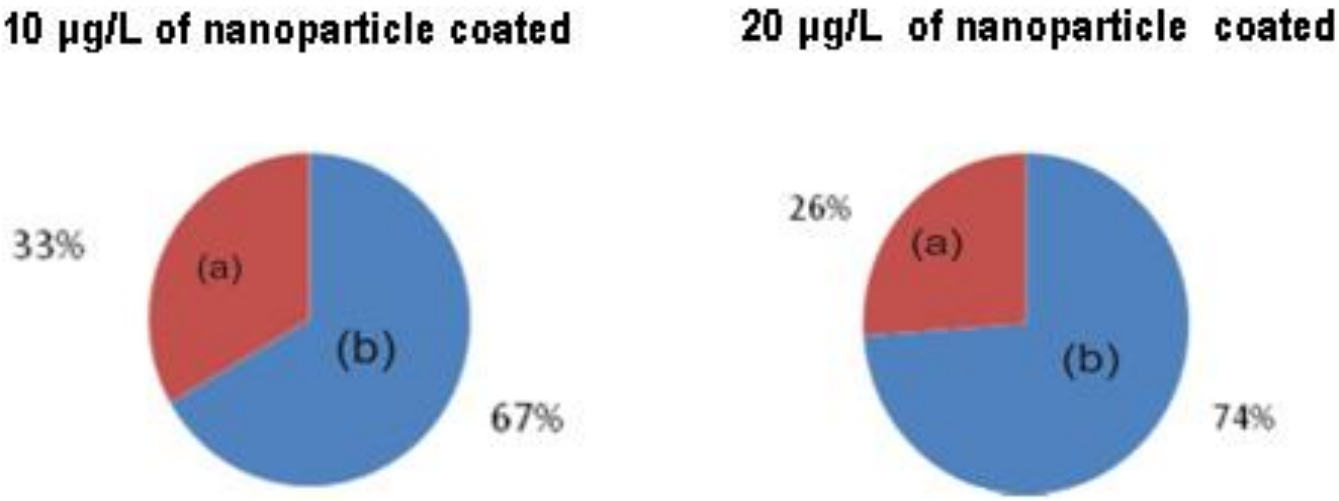


**Figure 6.** Pie chart zone of inhibition (mm) of uncoated (a) and flaxseed (b) iron oxide nanoparticles.

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