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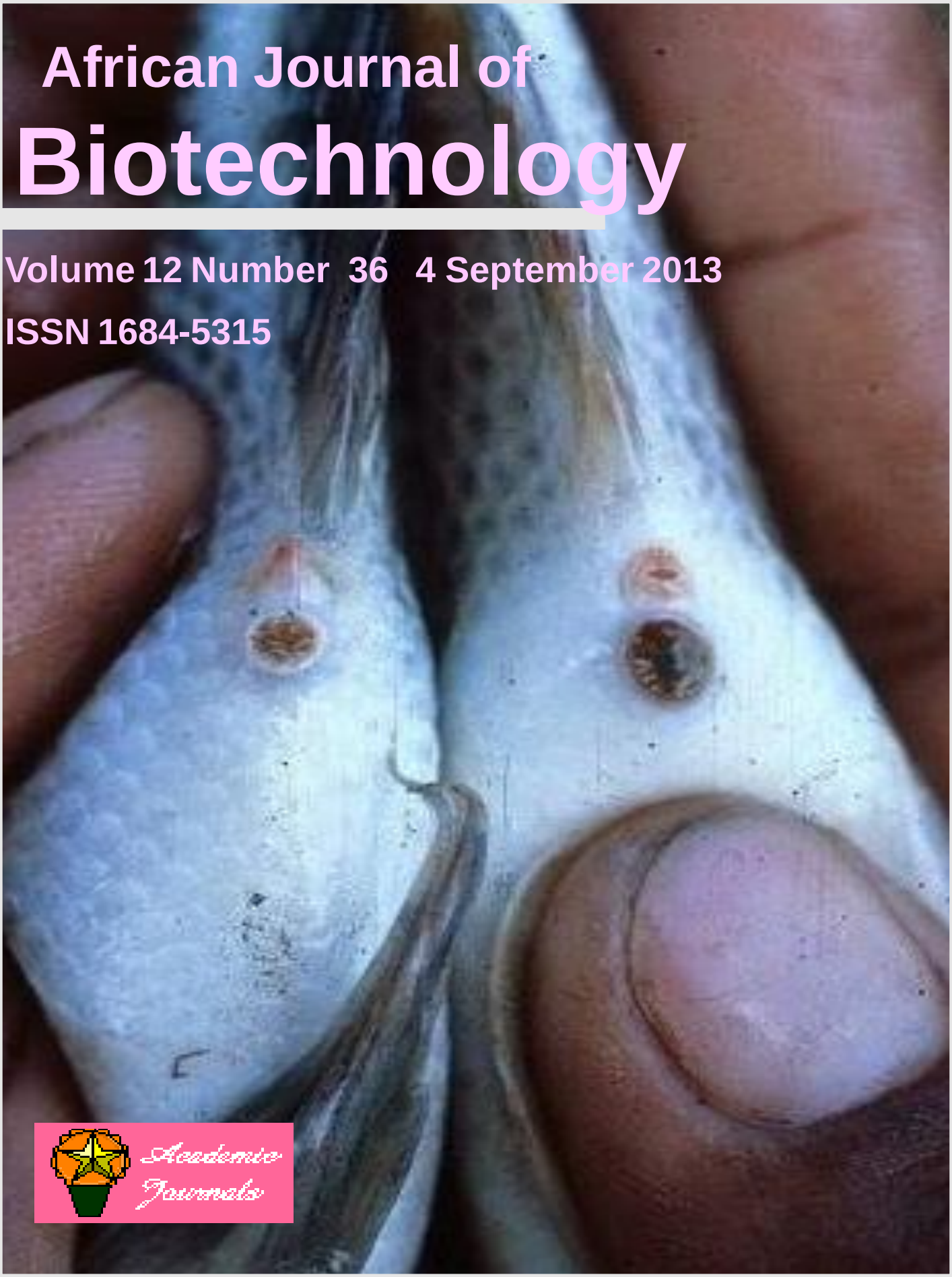

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Smith (2000), Blake et al. (2003), (Kelebeni, 1983), (Chandra and Singh,1992),(Chege, 1998; Steddy, 1987a,b; Gold, 1993,1995), (Kumasi et al., 2001)

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Pitter P, Chudoba J (1990). Biodegradability of Organic Substances in
the Aquatic Environment. CRC press, Boca Raton, Florida, USA.

Alexander M (1965). Biodegradation: Problems of Molecular Recalcitrance
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African Journal of Biotechnology

Table of Contents: Volume 12 Number 36 4 September, 2013

ARTICLES

Review

Research Articles

GENETICS AND MOLECULAR BIOLOGY

PLANT AND AGRICULTURAL TECHNOLOGY

Table of Contents: Volume 12 Number 36 4 September, 2013

ARTICLES

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Review

Nano lab-on-chip systems for biomedical and environmental monitoring

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In recent years, nano lab-on-chip (NLOC) has emerged as a powerful tool for biosensing and an active area of research particularly in DNA genetic and genetic related investigations. Compared with conventional sensing techniques, distinctive advantages of using NLOC for biomedicine and other related area include ultra-high sensitivity, higher throughput, *in-situ* monitoring and lower cost. This review aims to summarize recent advancements in two major types of NLOC sensing approaches, label and labelled free NLOC, as well as their biomedical applications. The state-of-the-art on how these sensors interface with nano/microfluidics is then presented and the latest papers in the area summarized and also proposal to develop compact NLOC with four different sensing elements with two different mechanisms, common and separate padding is prospected.

Key words: Nano lab-on-chip, *in-situ*, nano/microfluidics, sensors, DNA.

INTRODUCTION

In recent time, the quest for biological systems from molecules, through cells, to small multicellular organisms has explosively grown based on the advancement in nanotechnology (Kuo-Kang et al., 2010). This enabling technology allows sensing of ever-decreasing sample volumes and target analyte concentrations in ways that are not possible using conventional testing systems. Such technology also has the benefit of scaling the dimensions that enables a range of fundamental features to accompany system miniaturization such as reduced reagent consumption, high temporal resolution due to rapid response, high throughput, enhanced analytical performance, less waste, low unit cost, reduced energy consumption, and reduced dimensions when compared to microscale techniques (Squires and Quake, 2005; Kuo-Kang et al., 2010). It is a powerful tool holding great promise to facilitate novel experiments with unprecedented performance and has already found unique applications in chemical and system biology (Bringer et al., 2004; Breslauer et al., 2006; Weibel and Whitesides 2006), high-throughput biological screening (Hong et al., 2009), cell analysis and clinical diagnostic (Kuo-Kang et al., 2010; Sato et al., 2008) as well as point-of-care (POC) ion analysis for biomedical and environmental monitoring (Gardeniers and Berg, 2004). The significant development of bioanalysis and clinical analysis has mainly been driven by the strong demand for fast and reliable results, which are essential for early diagnosis and further medical treatment. Results concerning potential drug targets, vaccine studies and speciation of toxic substances must also be of the highest reliability (Kuo-Kang et al., 2010). These bioanalytical challenges in many cases can be solved using specifically designed and fabricated miniaturized tools called lab-on-a-chip systems or micro total analysis systems (μTAS) (Harrison et al., 1993). Advances in technology have allowed chemical and biological processes to be integrated on a single platform. Adaptation of these approaches to lab-on-a-chip or μTAS formats is providing a new kind of research tools for the investigation of biochemistry and life processes.

Tremendous effort and promising experimental results were established in lab-on-chip (LOC) research, however, the fundamental mechanism of the sensing unit design and the fluidic delivery system for the platform of the in-

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interaction remain poorly understood (Anderson and Berg, 2004; Jungkyu et al., 2009). For the fabrication of sensing element and the probing unit, alignment and exposure are the most critical steps in the process, the resolution requirements and precise alignment are vital; each mask needs to be precisely aligned with original alignment mark. Otherwise, it cannot successfully transfer the original pattern to the water surface causing device and circuit failure (Hong, 2009; Hashim et al., 2008). Precise transfer of pattern means guarantee in high repeatability and reliability, high throughput and low cost of ownership (Hong et al., 2008; Bien et al., 2009). By improving this resolution and alignment precision, the minimum size can be further reduced to 1 nm and beyond and other important aspect of achieving minimum precised size is that the photo resist must be very sensitive to the exposure light to achieve reasonable throughput. However, if the sensitivity is too high, other photoresist characteristics can be affected, including the resolution (Hong, 2009; Hallstedt et al., 2008; Ra et al., 2008). In the proposed design, we have designed 3 masks for the fabrication of poly-silicon nanowire (SiNWs) during pattern transfer process steps; each was precisely aligned with the previous to achieve successful pattern transfer in the fabrication. There was a very little room for alignment error; we were able to achieved less than 2% error of the critical dimension and for the sensing unit to reach its potential, it must be integrated with sophisticated fluid delivery system for complete diagnostic system, therefore, there is a need for microfluidics chip to be integrated with the sensing to achieve an affordable and reliable lab-on-chip.

LAB-ON-CHIP SYSTEM AND COMPONENTS

Systems

LOC systems for biosensing normally consist of a set of sensing unit, fluidic operation and probing units which allow different biomolecules to be detected and assayed in an easy and flexible manner. Overall, the chip-based platform which has good integration with micro/nanofluidic components is capable of detection for biomolecules.

Sensing unit

Nanoscale sensors have critical structural dimensions of less than 100 nm. Nanoscale transduction mechanisms are typically classified into two categories: label-based and label-free. Label-based transduction mechanisms typically rely on the presence of an added labelling molecule or structure and LOC sensing elements have been used to elucidate a wide spectrum of genetic and proteomic information with improved selectivity and sensitivity when compared to traditional methods. For example, nanotechnology-based, single-cell detection methods have been used to investigate how a cell responds to environmental changes, interacts with neighbouring cells, and expresses specific genes as it responds.

Label-free sensing method

Label-free methods have emerged as a potential way to avoid possible structural and functional alterations of target molecules while providing acceptable sensitivity and selectivity. Assay protocol simplification, which facilitates portable or POC sensing, is an additional expected advantage. With recent advances in micro and nanotechnologies, label-free biosensors have achieved attogram (Jungkyu et al., 2009; Jinquan et al., 2009) sen-sitivity and tremendous high-throughput analysis capabilities. Here, we mentioned the current state-of-the-art in label-free detection techniques, including nanowire, surface plasmon resonance, surface- enhanced raman scattering, micro/nano-cantilevers, and nanopores but for the purposes of this review, nanowire is fully described

Nanowire: SiNWs biosensors along with nanotube (Kong et al., 2000) and conducting-polymer nano wire (Ramanathan et al., 2005) are promising label-free electronic biosensors (Figure 1). The most important and powerful advantage of SiNWs sensors is the possibility of multiplexed and real-time detection. The progress in nanofabrication techniques allows us to make an array of identical structures, which leads to massively parallel measurements. As most nanofabrication techniques originated in microelectronics, they can be easily scaled-up and transferred to a mass production line with high reliability. The underlying mechanism of nanowire sensors is based on the principle of field-effect transistors (FETs). Figure 1a shows a p-type SiNWs FET and its response to different gate voltages. In the case of a p-type semiconductor, a positive gate voltage depletes carriers and reduces the conductance, while a negative gate voltage leads to an accumulation of carriers and an increase in the conductance. For biosensors, binding of a charged species on the surface of the SiNWs is analogous to applying a gate voltage. By monitoring the conductance change, the binding of targets to probe molecules can be detected on the Si surface. Several research groups have already demonstrated the successful solution-phase SiNWs sensing of DNA viruses (Patolsky et al., 2004), small molecules (Wang et al., 2005) and proteins (Stern et al., 2007a; Zheng et al., 2005). The current detection sensitivity of fibromyalgia (fM) range is, in several orders of magnitude, more sensitive than a conventional enzyme-linked immunosorbent assay (ELISA) assay. For most of those experiments, however, low salt buffer solutions were used to avoid the screening effect associated with solution counter ions (Stern et al., 2007b). The charge of target molecules is screened by the counter ions in solution and effective only on the scale of the Debye length.

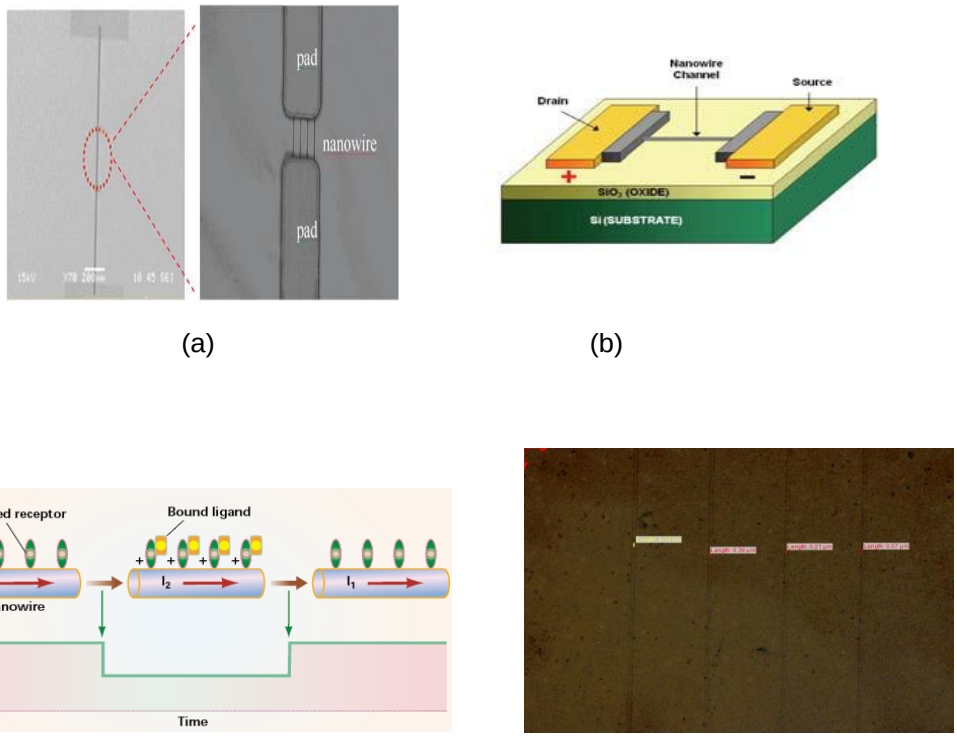

Figure 1. (a) Schematic of a regular p-type ET device. S and D correspond to source and drain; (b), HPM image observed the connected nanowire between the two electrode pads and the 3-D model of overall pattern fabricated on the sample; (c), operation principle of nanowire filled with DNA (Uda et al., 2008); (d), the SEM image of the silicon nanowire (Jungkyu et al., 2009).

Several research groups recently focused on the importance of the Debye length in this application (Stern et al., 2007b; Zhang et al., 2008). The Debye length for a 0.1 M solution is about 1 nm and biologically relevant media is typically a 0.14 M electrolyte. Since the salt concentration and pH are important factors for the binding between biomolecules, it is necessary to find an alternative way of overcoming the charge screening to perform an ideal sensing measure-ment with NW-FETs (Huan-Xiang, 2001). For DNA sensing, the charge screening can be overcomed by electro statically immobilizing ssDNA on the SiNWs surface (Bunimovich et al., 2006). With antibodies, however, the bimolecular recognition event occurs at 10 nm away from the wire due to the antibody's large size. In biological media, the binding event usually takes place farther away than the Debye screening length; so alternative small capture agents are required to bring the binding event closer to the nanowire (Figure 1c). Thus, finding small molecules that have the same specificity for proteins as antibodies and that can distinguish between slightly different proteins is critical. Due to their small scale, high sensitivity and real-time detection capability, nanowire based sensors could be used to study single cells (Figure 1d).

Label-based sensing method: Biomolecules such as proteins and nucleic acids often cannot be recognized directly due to their small size. To track these biomolecules and their activity, probes for these target molecules or the target biomolecules themselves can be labelled by conjugation with a detectable agent, commonly a fluorophore or an enzyme. Labelling methods allow high sensitivity and these approaches are developed to the point that they give reproducible results. These agents for labelling proteins, nucleic acids, and other molecular probes are called tags. These tags have unique detectable properties such as radioactivity, chromogenicity, fluorescence, or magnetism. Additionally, electrical and electrochemical principles, based on the properties of labelled probes have been developed to establish corresponding detection methods through a target binding technique (Figure 2). Having a uniquely detectable property, most tags can be functionalized to link to a specific molecular probe. In a related approach, instead of having a detectable group directly attached to a probe molecule, a recognition reagent having strong affinity for a secondary probe can be used to detect the target molecules. A variety of interaction pairs, such as biotin-avidin, hapten-antibody, and DNA–RNA hybrids, etc., are already in use (Kessler et al., 1992; Jungkyu et al., 2009). This two-stage detection scheme can be utilized when a primary-labelled probe is not available (Jungkyu et al., 2009). A schematic diagram for both labelling approaches is shown in Figure 2a. In the past, scientists mostly relied

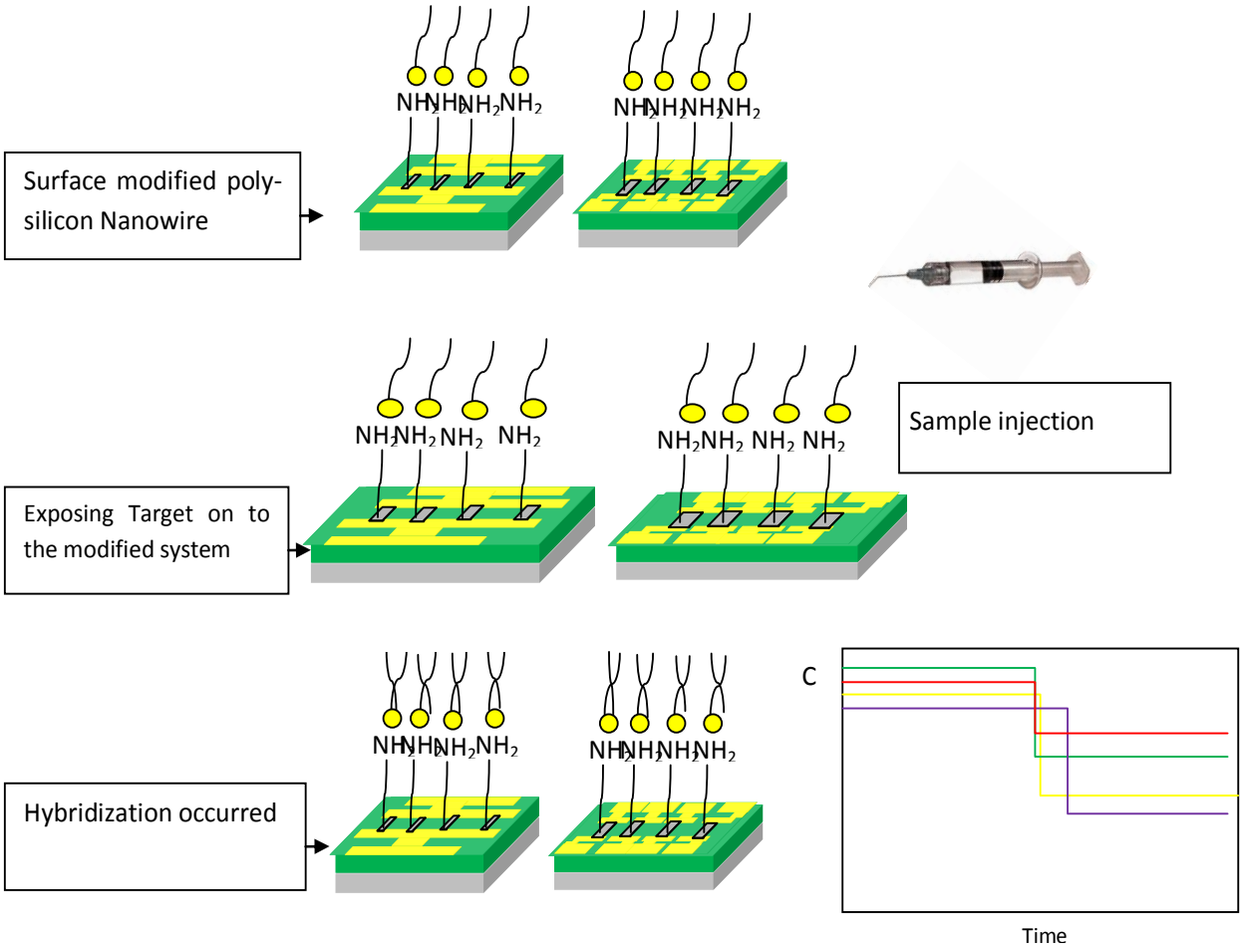

Figure 2. (A) Schematic diagram of two approaches to label-based biomolecules detection: (a), Direct method; (b), indirect method; (B), schematic diagram of nano-shell (quantum dot) detection by attaching probe molecules to the nanoparticles surface; (C), schematic diagram of using a gold nanoparticles as a label and possible detection methods (Jungkyu et al., 2009).

on radioactive probes to detect samples. However, safety and convenience concerns spurred the development of alternative techniques (Gary, 1993). Among a range of options, current bio-molecules detection methods have mainly employed fluorescent labels, quantum dots, or heavy atom complex nano-particles labels. Chromogenic labels are also available, but they have been replaced with fluorescent labels which give larger quantum emission yield upon excitation resulting in better delectability (Jungkyu et al., 2009). Bioluminescence, which generates detectable light as a result of biochemical reactions, is another popular technique for bio detection. Other detection methods are Au nanoparticles and magnetic nanoparticles labels. These techniques are emerging as better substitutes in terms of simplicity, sensitivity, specificity and reliability for the current standards.

Fluid delivery unit

Microfluidic systems for biosensing normally consist of a set of fluidic operation units that allow different biomolecules to be detected; the chip is capable of sampling, filtration, pre-concentration, separation, restacking, and detection for biomolecules and based on their flow type, microfluidic system can be categorized into two main types, continuous and discrete. Continuous-flow microfluidic operation is a promising approach because it is easy to implement and less sensitive to protein fouling problems. Continuous-flow devices are adequate for many well-defined and simple biochemical applications, and for certain tasks such as chemical separation, but they are less suitable for tasks requiring a high degree of flexibility or complicated fluid manipulations. These closed-channel systems are inherently difficult to integrate and scale because the parameters that govern the flow field vary along the flow path making the fluid flow at any one location dependent on the properties of the entire system. Permanently-etched microstructures also lead to limited reconfigurability and poor fault tolerance capability. In other words, droplet-based microfluidic systems are currently an emerging area of microfluidic research. One of the most popular means is to inject multiple laminar

streams of aqueous reagents into an immiscible carrier fluid and therefore to induce flow instability instantly for forming the droplets (Tice et al., 2003). There are several distinctive advantages based on droplet-based microfluidic systems. First, the systems promise a new high-throughput technology that enables the generation of micro droplets in excess of several thousand per seconds (Huener et al., 2007). In addition, parallel and serial *in vitro* compartmentalization is possible with this technology. The reagents are con-fined inside the droplets in water-in-oil (w/o) emulsions and reagent transport occurs with no dispersion (Kelly et al., 2007). The fluidics components consist of micropump, valve, micromixer, separator and concentrator. Among these components, micropumps and micromixer are the key components for microfluidic applications due to their actively functioning capability. Therefore, for the purpose of this review, the micropumps and micromixer are fully described.

Micropump

Controlling fluid flow is crucial in microfluidic devices, especially for processing biochemical reactions. Such a process generally relies on active control by mechanical pressure (Linder et al., 2005; Marmottant and Hilgenfeldt, 2004), electroosmotic force, electro wetting (Jun and Kim, 1998); Huh et al., 2003) and electrochemical reaction (Gallardo et al., 1999). These active manipulations enable close control in a rapid and precise manner. Electrokinetic sampling has been widely used for microfluidic chip, especially for microfluidic chip electro-phoresis, because the electric field can be easily and precisely applied to the reservoirs on the chip. The popular mechanism used for these active micropumps is electrokinetic force. Based on the mechanism, various micropumps such as dielectrophoresis, asymmetric electric field, electroosmosis and electrophoresis (the latter two are considered as part of the electrohydrodynamic (EHD) phenomena) (Green et al., 2000; Brown et al., 2000) have been developed. Moving sample fluids and reagents on a biosensing microfluidic device requires developing a pressure difference in the flow path to direct fluid in one direction or another. Miniaturized versions of positive-displacement pump designs such as gear or peristaltic pumps have been proposed for microfluidic applications, but these all require some external power source or repetitive motion to control. It is desirable for fluidic motion in a passive microfluidic system design to be driven by a readily available force such as gravity, capillary action, absorption in porous materials, chemically induced pressures or vacuums (for example, by a reaction of water with a drying agent), or by vacuum and pressure generated by simple manual action. Wicking and capillary action have been widely used to motivate fluids for POC diagnostics. For example, low cost lateral flow tests demonstrate the elegant and inexpensive use of wicking to drive multiple sample types through all steps of an assay. One of the simple methods for transporting fluids on microfluidic devices is to apply pressure manually to deflect a diaphragm (Moorthy et al., 2004). Diaphragm membrane pumps have been demonstrated successfully in moving fluid on a microfluidic device. However, it is not easy to control the flow rate in a reproducible way. Zhu et al. (2002) reported a gravity microfluidic pump for producing constant flow rate. This passive system employs a microchannel and a gravity-driven pump consisting of horizontally oriented reservoirs that supply fluid to the microchannel at a substantially constant rate. The passive device may be useful for numerous microfluidic applications such as cell-size sorting (Huh et al., 2002). The pumps have been developed based on osmotic pressure as the actuation mechanism have been used in many drug-delivery applications to deliver medication over a prolonged period of time (Su et al., 2002; Su and Lin, 2004). The advantages of these pumps include simple construction and the absence of moving parts. Another passive system involves controlled evaporation of a liquid into a chamber with an absorption agent flow (Effenhauser et al., 2002). As fluid evaporates from the channel, capillary forces induce fluid flowing from reservoir to replace the evaporating fluid. This micropump has advantages of low cost, high reliability and constant flow rate over a long period of time. The major disadvantage of the evaporation micropump is the need to control environmental conditions for constant flow rates and lower flow rates. The micropumps have also been developed by employing fluid-responsive polymers to deliver fluids (Eddington and Beebe, 2004). Fluid-responsive polymers swell when exposed to certain environmental conditions, such as changes in moisture, pH or temperature. One recent fluid-responsive pump consists of an array of responsive polymers that deforms a flexible membrane made from polydimethyl siloxane (PDMS) and produces flow rates (Eddington and Beebe, 2004). The disadvantage of the pump is the requirement of pressure to inject the buffer solution in order to active the pump.

Micromixer

Mixing is a physical process to achieve homogeneity of the different components involved in certain process. In some cases, the mixing will be the rate determining step when the mixing time is in the same order or longer than the molecular reaction time. Because the fluid streams mainly appear naturally as laminar flow on a chip, the mixing will mainly depend on molecule diffusion. Mixing small amounts of reagents and samples in microfluidic channels or structures is a challenging task. Likewise, mixing in passive micromixer relies mainly on molecular diffusion and chaotic advection. To speed mixing process, the T-mixer or Y-mixer which consists of the inlets converging into a long microchannel has been developed as a simple and effective solution (Kamholz et al., 1999; Wang et al., 2005; Kamholz and Yager, 2003; Ismagilov et al., 2000). Other methods for fast mixing have been implemented through reducing the mixing path in a narrow

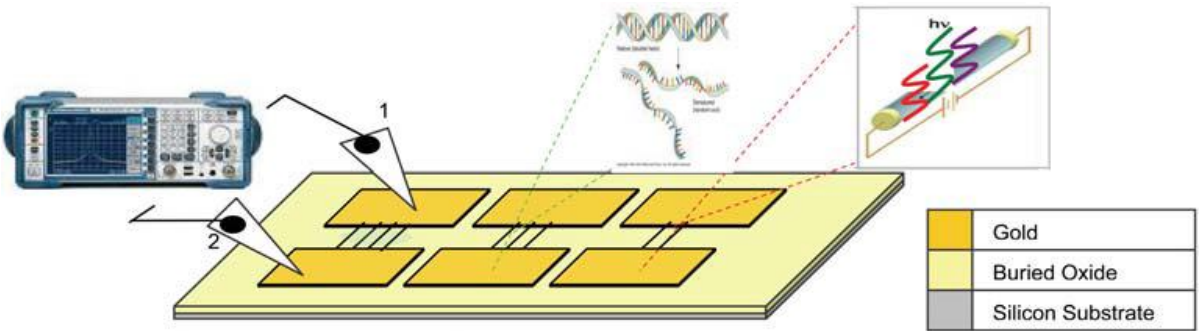

Figure 3. After gold pad formation, measurement of different nanowire pattern using spectrum analyzer semiconductor parameter analyzer are taken (Uda et al., 2008).

mixing channel (Veenstra et al., 1999) and realizing parallel lamination with multiple streams (Jackman et al., 2001; Koch et al., 1999). Besides diffusion, advection is another important form of mass transfer in flows with a low Reynolds number. However, advection is often parallel to the main flow direction, and is not useful for the transversal mixing process. The chaotic advection generated by special geometries in the mixing channel can improve mixing significantly. The basic idea is the modification of the channel shape for splitting, stretching, folding and breaking of the flow. The simplest method to get chaotic advection is to insert obstacles or structures in the mixing channel. However, it has been shown that eddies or recirculation cannot be generated in a microchannel, because of its low Reynolds number (Wang et al., 2002). The effective method to produce chaotic advection is to modify the wall of mixing channel with ribs, grooves and staggered-herringbone grooves. Johnson et al. (2002) were the first to investigate this phenomenon. They ablated the grooves on the bottom wall of the channel by laser. This structure allows mixing at a relatively slow velocity of 300 μm/s. Stroock et al. (2002) investigated two different groove patterns, slanted groove and staggered. The so-called staggered herringbone mixer can work well at low Reynolds number

Probing unit

Prior characterization and electrical testing, a process called metallization is done; a variety of conductors is applied in chip fabrication. Metal with high conductivity are widely used for interconnections forming microelectronics circuit. Metal such as copper, aluminium and gold are good conductors and are widely used to make conducting lines to transport signal; the requirements for metallization are low resistivity for low power consumption and quick device response, smooth surface for high resolution patterning process, high resistance to electro migration to achieve high device reliability, and low film stress for good adhesion to underlying substrate and other requirement are stable mechanical and electrical properties during subsequent processing, good corrosion resistance and relative receptivity to deposit and etch.

It is important to reduce the resistance of the inter-connection lines, since the chip speed/response is closely related to the RC time, which is proportional to the resistivity of the conductor used to form the metal line. The lower the resistivity, the shorter the RC time and the faster the chip. For the purpose of the review, presented here is focus on gold. A report from Uda et al. (2008) stated that a contact point is formed by deposition of aurum (gold) material prior to the fabricated nanowire. Gold is used to have a good reliability via contact and it has a very good conductivity. This is to ensure that the device has a good electron flow and no bias effect to the sensing nanowire. A layer of 500 nm thick of aurum is deposited using E-beam evaporator onto the surface of the fabricated nanowire. The layer is then coated and patterned using photolithography process to form the contact point. *Aqua Regia* is used for etching. Finally, the photoresist layer is removed to expose the gold pad for contact (Figure 3).

Prospect and the summary of the work

The SiNWs based biosensor fabrication process comprises of three major steps namely wire formation, wire trimming and gold pad formation. In development of nano lab-on-chip (NLOC), the sensing unit is very crucial and here we presented some critical parameters to be considered during fabrication of nanowire for biosensing, thus, alignment and exposure are the most critical steps in photolithography process, the resolution requirements and precise alignment are vital; each mask needs to be precisely aligned with original alignment mark. Otherwise, it cannot successfully transfer the original pattern to the wafer surface causing device and circuit failure. Precise transfer of pattern transfer means guarantee in high repeatability and reliability, high throughput and low cost of ownership. By improving this resolution and alignment precision, the minimum size can be further reduced to 1 nm and beyond and other important aspect of achieving

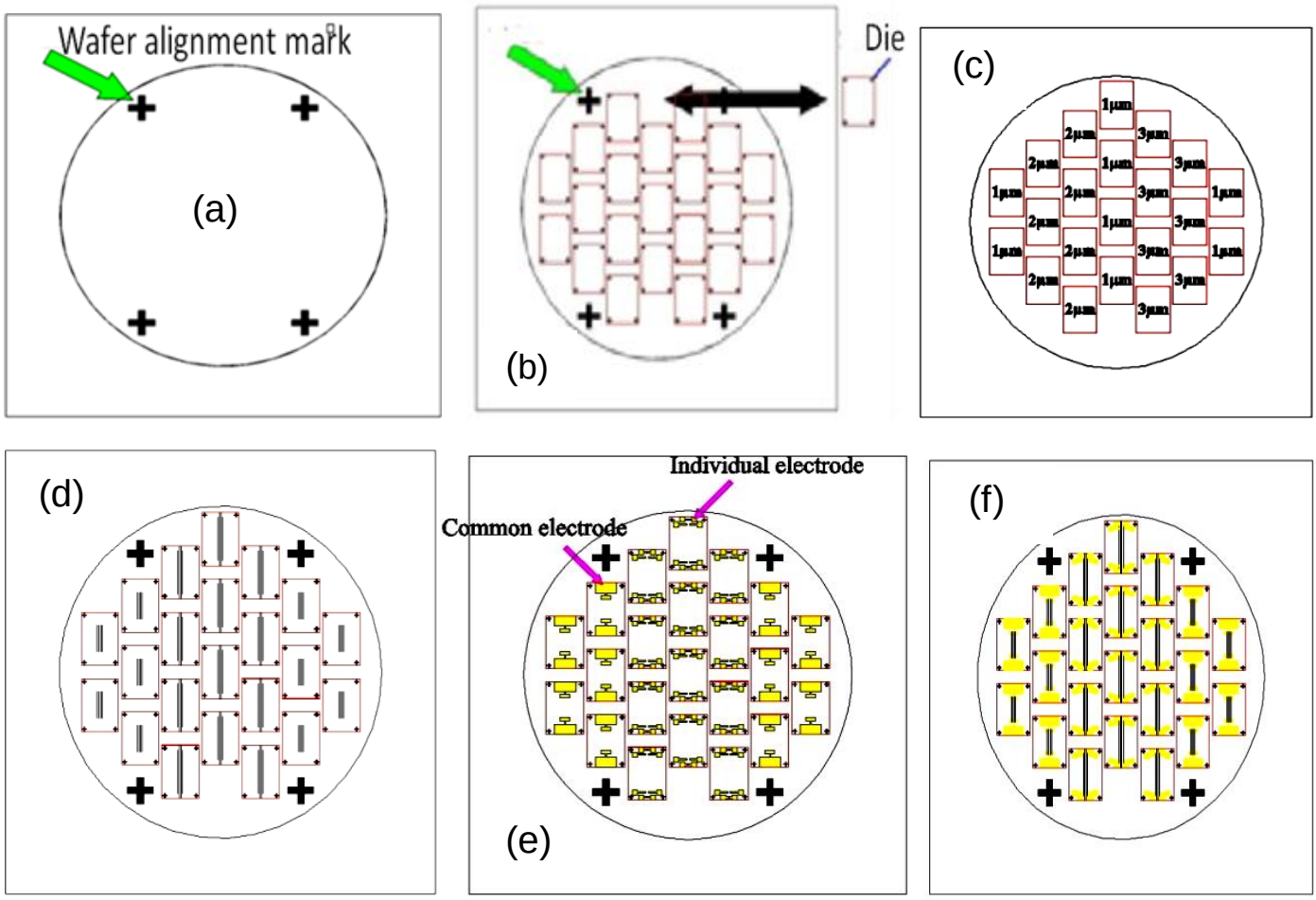

Figure 4. Mask design layouts. (a), Wafer alignment mark; (b), die alignment mark; (c), die position based on wire dimensions: 1, 2 and 3 µm; (d), wire mask layout; (e), common and individual electrode mask layout; (f), complete mask layout design.

minimum precised size is, the photoresist must be very sensitive to the exposure light to achieve reasonable resolution. In this study, we have 15 pattern transfer process steps to fabricate nanowire; each was precisely aligned with the previous to achieve successful pattern transfer in our design. There was a very little room for alignment error; we were able to achieved error free design to the critical dimension shown in Figures 4, 5 and 6

After poly-SiNWs, the fabrication process was completed, and then the fluidic chamber fabrication follows. This also consists of two major steps; started with the master template development and followed by replica fabrication of chamber and inlet/outlet channel. Subsequently, the inspection and characterization of the fabricated devices were conducted using transmission electron microscope (TEM), focused ion beam (FIB), field emission scanning electron microscope (FESEM) and atomic force microscope (AFM) instruments. After the testing of individual device was accomplished, the integration of the fabricated devices was done using plasma oxidation process with the two major integration steps throughput. However, if the sensitivity is too high, other photoresist characteristics can be affected, including the namely surface treatment and device mounting (Figure 7). Finally, SiNWs surface modification and DNA immobilization for DNA hybridization was done on SiNWs LOC for the testing and validation through electrical testing by using real biological samples. The integrated NLOC design with two probing approaches, in the common gold pad probing, the probe respond to any of the four transducer response and for the individual probing. As shown in Figures 7a and b, it has four different wires connected to the individual transducer and it means that, it responds individually for changes it experience from nanowire.

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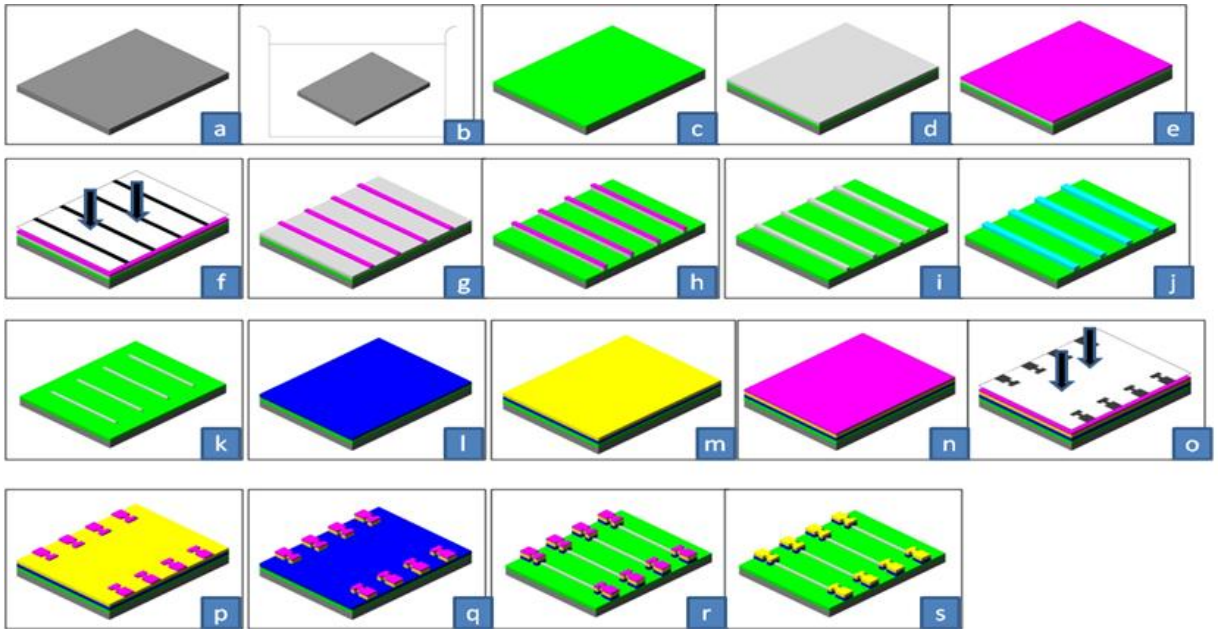

Figure 5. Fabrication process flow of polysilicon nanowire with common electrode. (a), Silicon wafer; (b), wafer preparation; (c), wafer insulation (Si_3N_4); (d), polysilicon deposition; (e), photoresist coating; (f), alignment and exposure; (g), resist development; (h), resist striping; (i), polysilicon microwire; (j), trimming process by plasma oxidation; (k), polysilicon nanowire; (i), titanium (Ti) deposition for metallic connection; (m), gold deposition for contact formation; (n), photoresist coating; (o), alignment and exposure; (p), resist development; (q), gold etching; (r), resist stripping; (s), Individual electrode polysilicon nanowire.

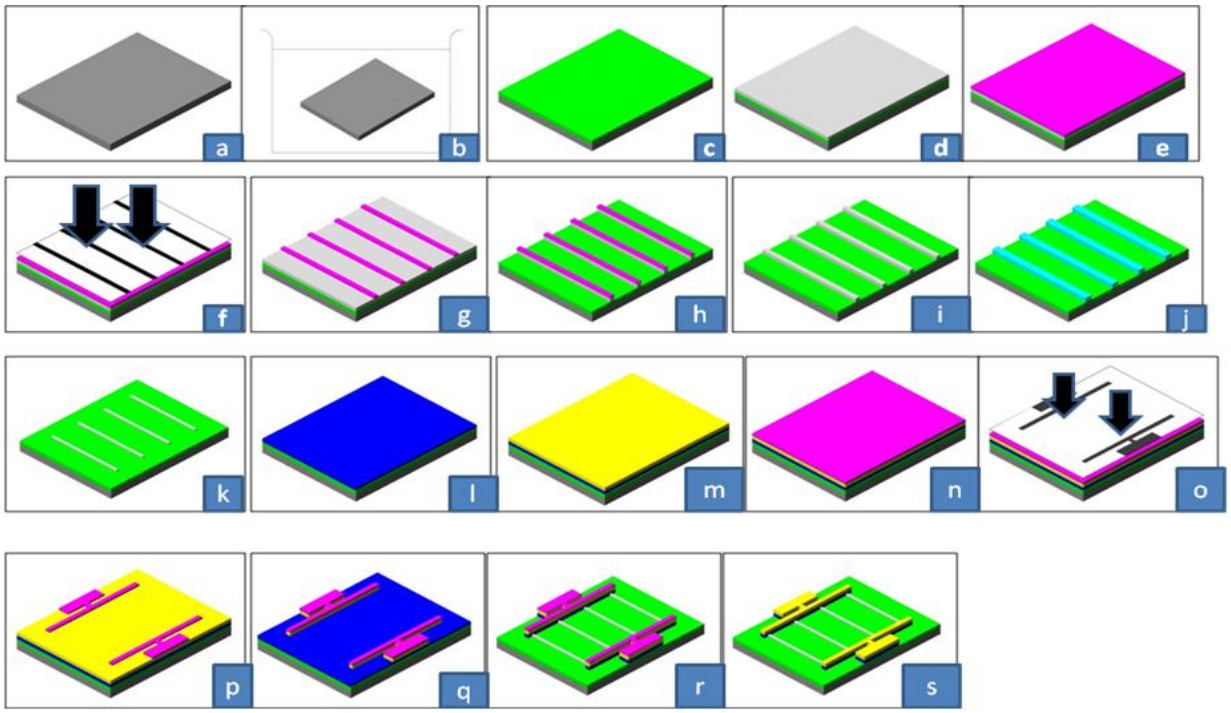

Figure 6. Fabrication process flow of polysilicon nanowire with common electrode. (a), Silicon wafer; (b), wafer preparation; (c), wafer insulation (Si_3N_4); (d), polysilicon deposition; (e), Photoresist coating; (f), alignment and exposure; (g), resist development; (h), resist striping; (i), polysilicon microwire; (j), trimming process by plasma oxidation; (k), polysilicon nanowire; (i), titanium (Ti) deposition for metallic connection; (m), gold deposition for contact formation; (n), photoresist coating; (o), alignment and exposure; (p), resist development; (q), gold etching; (r), resist stripping; (s), Individual electrode polysilicon nanowire.

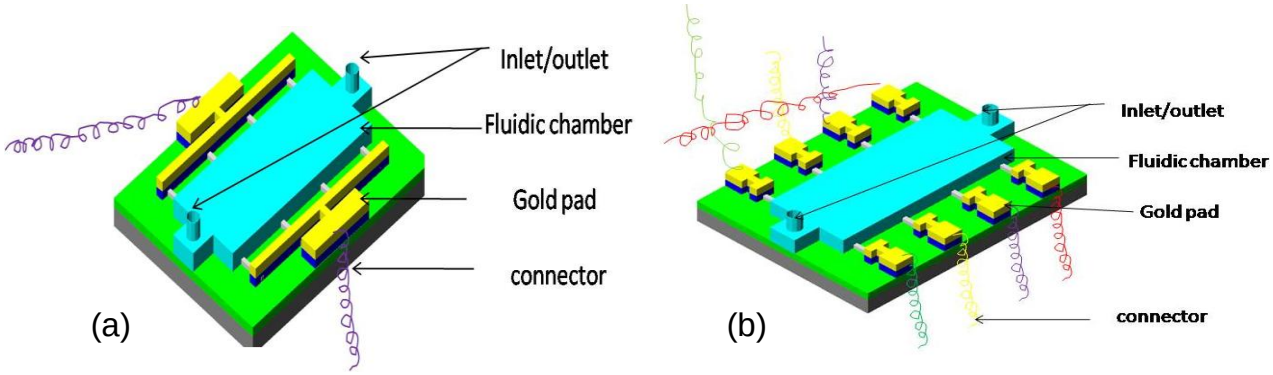

Figure 7. Integrated nano lab-on-chip. (a), Common gold pad probe device; (b), individual gold pad probe.

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Review

Male tilapia production techniques: A mini-review

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Tilapia culture has been growing over the past decades as an excellent source of high-quality protein. Some of the Tilapia´s advantages are the ability to breed and produce new generations rapidly, tolerate shallow and turbid waters, resist a high level of disease and be flexible for culture under many different farming systems. These characteristics are the main reasons for its commercial success. However, one of them contributes to the major drawback of pond culture: the high level of uncontrolled reproduction that may occur in grow-out ponds. Uncontrolled reproduction yields to stunted growth and unmarketable fish due to offspring competing with the initial stock for food, besides other problems like less dissolved oxygen, greater release of ammonia and feces, heterogeneous sizes and overpopulation stress. Monosex production has been preferred in order to deal with these issues. Males are preferred because they grow almost twice as fast as the females. This paper reviews monosex male production techniques and their results, comprising environment manipulation, hybridization, sex reversal and genetic manipulation. The choice of a particular technique would depend on the legislation of each country. This review's should help to select the appropriate technique depending on the market target and the commercial technology available.

Key words: Monosex production, hybridization, sex reversal, environmental and genetic sex determination.

INTRODUCTION

Mainly taking the form of fish farming, aquaculture has skyrocketed in the past three decades. It is growing at 9% annually and is projected to contribute 41% (53.6 million tonnes) of the world's fish production by 2020 (Krishen et al., 2009). Today, low-income food-deficit countries, mostly in Asia, account for nearly 85% of the world's aquaculture production. Scientists began their research by focusing on Nile Tilapia because of its ability to breed and produce new generations rapidly, its tolerance for shallow and turbid waters, its high level of disease resistance and its flexibility for culture under many different farming systems (Yosef, 2009; Soto-Zarazúa et al., 2010a). The major drawback of pond culture is the high level of uncontrolled reproduction that may occur in grow-out ponds. Monosex culture is one the basic methods of controlling Tilapia populations that have been carried out in some countries for aquaculture purposes. This technique includes manual separation of sexes, environmental manipulation, hybridization, hormone augmentation (sex reversal) and genetic manipulation methods such as androgenesis, gynogenesis, polyploidy and transgenesis. None of these methods is consistently 100% effective, and thus a combination of methods is suggested.

Males are preferred because they grow almost twice as fast as females, which may be caused by a sex-specific physiological growth capacity, female mouth-brooding or the more aggressive feeding behavior of males. Expected survival for all-male culture is 90% or greater. A disadvantage of male monosex culture is that fingerlings have to be grown until it is possible to distinguish the female and male juveniles (at least up to 50 g) and then the female juveniles are discarded. The percentage of females mistakenly included in a population of mostly male Tilapia affects the maximum attainable size of the original

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Figure 1. Tilapia sex differentiation (photo by Rob. L. Elliott).

stock in grow-out phase. The density for male monosex culture varies from 10,000 to 50,000/ha or more, at proper feeding rates. Densities of around 10,000/ha allow the fish to grow rapidly without the need for supplemental aeration. About six months are required to produce 500 g fish from 50 g fingerlings, with a growth rate of 2.5 g/day (Fortes, 2005). A sex (male or female) is heterogametic if it has two different sex chromosomes (XY) and is homogametic if it has a matching pair of sex chromosomes (XX or YY). Without external influence Tilapia sex will be determined genotypically as female if it has two X chromosomes and will be male if it has XY chromosomes. Fish have certain plasticity during sex differentiation since several functional sex phenotypes can be generated by diverse mechanisms (Baroiller and D'Cotta, 2001). Thus, we can obtain phenotypically XX sex-reversed males and YY supermales.

Male Tilapia production has an economic importance to its producers and sellers. The increase in employment in the sector outpacing world population growth and employment in traditional agriculture is a crucial source of income and livelihood for hundreds of millions of people around the world (Soto-Zarazúa et al., 2011). It could play an important role to provide food security for the general population as an excellent source of high-quality protein (FAO, 2010; Soto-Zarazúa et al., 2010b). The aim of this review was to help choose a particular technique for the male Tilapia production based on a comparison between the techniques currently available, including the traditional techniques such as manual sorting and hybridization, to high-tech techniques which modify the genetic structure of the fish to produce male Tilapia.

MANUAL SORTING

Some species of the genus Tilapia can be easily sorted into males and females. Either the colors are sufficiently differentiated to serve as reliable sex indicators or the structure of the anal papilla is used; the opening of the oviduct is distinguishable in the female and is not present in the male as shown in Figure 1. Turning the fish over, it is possible to look at the secondary sex organs (shaped like a small cone) located behind the anus. Male tilapia (left on Figure 1) has a simple papilla, while the female (right on Figure 1) have a slightly wider organ with a wide opening to allow eggs to eject during mating. To make it easier to distinguish the ovarian opening, it is possible to use a dye such as gentian violet. Using a cue tip that is dipped in a violet dye and smeared lightly over the papilla from front to back, the dye outlines the openings while improving visibility.

With experience it is possible to notice the sex of even small immature fish with speed even though human error is always present. A second check is made when the fish have grown somewhat larger and distinctive sex-coloration is more discernible. Since this technique fails if there is a single female present in the raising pond, care must be taken to ensure that there are no females left over from a previous stocking. The sexing of small Tilapias, although feasible, is tedious and not entirely reliable (Hickling, 1963). Additionally, it is stressful for the fish. While it is an easy technique, it is extremely laborious and human accuracy varies from 80 to 90%, which leads to the presence of females in the pond. Therefore, this method is rarely used (Penmann and McAndrew, 2000). Moreover, this technique may be useful in small populations, but in commercial practice their use increases the cost of skilled labour and increases the risk of human error, leading to uncontrolled reproduction.

ENVIRONMENTAL MANIPULATION FOR SEX DETERMINATION

Tilapia is a thermo-sensitive species and its male to female ratio increases with temperature and/or ovarian differentiation is induced by low temperatures. Fish show particularities in their temperature sex determination patterns since monosex populations are generally not produced at extreme temperatures, suggesting the existence of strong temperature genotype interactions. Temperature treatments must be applied at a critical sensitive period, relatively similar to the hormone sensitive period. Molecular mechanisms of thermo-sensitivity could be addressed in Tilapia species (example *Oreochromis niloticus*), where aromatase gene expression is down-regulated by masculinising temperature treatments. Furthermore, in Tilapia, the gene expression of 11β-hydroxylase (a key enzyme involved in the synthesis of 11-oxygenated androgens) does not appear to be affected by temperature treatments (Baroiller and D´Cotta, 2001).

Table 1. Hybridization to produce all-male progeny.

Crosses (female x male)	Result
O. niloticus × *O. variabilis* *O. nigra* × *O. urolepis hornorum* *O. vulcani* × *O. urolepis hornorum* *O. vulcani* ×*O. aureus*	98 to 100% (Fishelson, 1962; Pruginin, 1967; Pruginin et al., 1975; Hsiao, 1980; Hulata et al., 1983, 1993)
O. niloticus × *O. urolepis hornorum*	All-male progeny (Wohlfarth et al., 1990)
O. niloticus × *O. aureus*	All-male progeny (Wohlfarth, 1994)

Meanwhile, a strong effect of temperature on sex differentiation has been demonstrated in various Tilapia species and in a hybrid (Baroiller et al., 1995a, b; Baroiller and Clota, 1998; Desprez and Mélard, 1998; Wang and Tsai, 2000). Baroiller et al. (1995a) demonstrated that Tilapias were sensitive to temperature during the critical period of sex differentiation. It was possible to masculinise XX progenies (100% females) with elevated temperatures above 32°C, giving functional male phenotypes. High temperatures could efficiently masculinise some progenies if started around 10 days post fertilization and if applied for at least 10 days, with longer periods being just as effective (Baroiller et al., 1995a, b; Tessema et al., 2006; Wessels and Hörstgen-Schwark, 2008). However, if a treatment was applied for a 10-day period but begun at 7 days post fertilization, it had no effect on sex ratios (Baroiller et al., 1995a, b). This window for temperature sensitivity coincides with the gonad sensitivity towards other external factors, notably hormones. Like temperature, hormonal treatments or the use of aromatase inhibitors during sex differentiation can override the genetic sex determination, inversing sex and producing functional phenotypes (Nakamura, 1975; Baroiller et al., 1999; Guiguen et al., 1999).

The temperature sensitivity of Nile Tilapia during sex differentiation is not seen in all progenies and can be heritable in Nile Tilapia (Wessels and Hörstgen-Schwark, 2008). Together, these studies showed that sex in the Nile Tilapia is governed by the interactions of three components, a complex genetic sex determination system with a major determinant locus and some minor genetic factors, as well as the influence of temperature. Unfortunately most of these studies showing sensitivity to temperature are hindered because the sex determination mechanisms of most of these species have not been able to be well characterized and physiological, genetic or ecological studies cannot be carried out to better understand the component of this environmental sensitivity (Baroiller et al., 2009). Although studies are underway, this technique is not reliable due to multiple variables that need to be taken into consideration and there are other treatments that are simpler and achieve better results.

HYBRIDIZATION

Hybridization takes advantage of qualitative variances to improve genetics in Tilapia by crossing two closely related but distinct subspecies of fish. If the sex determination system is different, the hybridization between a female homogametic and a male homo-gametic produces only male offspring (Wohlfarth and Hulata, 1991; Trombka and Avtalion, 1993). Hybrids from Oreochromis with two opposing "sex chromosomes" models (XX/XY in *O. niloticus* and *O. mossambicus* and WZ/ZZ in *O. aureus* and *O. urolepis hornorum*) exemplify the complexity of sex determination model in their pure species. Wohlfarth and Hulata (1991) suggests that the genetic mechanism of sex determination in Tilapias is analogous to that in platy fish, depending on variation in both sex chromosome and autosomally carried factors. Empirical evidence for this is not available for Tilapias due to the absence of sex-linked markers. In such a complex system, the chances of producing “super males”, which generate all-male broods with any female, in either intraspecific combination, appear small. A super male Tilapia (YY) could be attained by feminizing genetic males (XY) with estrogens and then breeding them with normal males (XY), which leads to three different possibi-lities: females (XX), males (XY) and super males (YY). This strategy has various limitations to consider. To create an YY bank, several generations have to be analyzed and the technique is not 100% effective, which implies the possibility that other factors directly influence sexual determination (Green et al., 1997).

There are more than 100 tilapia species but the most prominent for aquaculture are the Nile tilapia (*O. niloticus*), the Mozambique tilapia (*O. mossambicus*), and the blue tilapia (*O. aureus*) *O. niloticus* or one of its subspecies are commonly preferred in tropical freshwater while *O. aurea* has increased cold tolerance so it is grown in subtropical freshwater. Table 1 shows some of the crosses that lead to all-male progeny and their best results reportted. Among the major constraints in producing hybrids are: maintaining the purity of brood stocks, limited fecundity of parent fish which restricts fry production and diffi-

culty in producing sufficient number of hybrid fry due to spawning incompatibilities between the parent species. In as much as not all crosses produce 100% males, the hybrids may still be subjected to manual separation of sexes or hormone augmentation. Some advantages of hybridization are that it saves time, space and feeds, but it is not a perfect solution (Fortes, 2005).

SEX REVERSAL OR HORMONE AUGMENTATION

This method can be performed by oral administration of feed incorporated with androgen and eggs or fry immersion in different concentrations of the male hormone. The principle behind this method lies on the fact that at the stage when the Tilapia larvae are said to be sexually undifferentiated (right after hatching up to about 2 weeks or up to the swim-up stage), the extent of the androgen (male hormone) and the estrogen (female hormone) present in a fish is equal. Thus, augmenting one of the hormones that is originally present in the fish will direct the fish to either male or female depending upon the hormone introduced. Accordingly, if the Tilapia larvae are fed with feeds that are incorporated with male hormone as example 17α-methyltestosterone, the fish will develop into phenotypic male physically and function as male but possess the female genotype (XX). In the same way, if a female hormone is mixed with the feed that is taken by the fish, then the fish will be directed to phenotypic female physically and functions as female, but possesses the male genotype (XY). This is commonly referred to as "sex reversal".

Different steroids have been used over the years to induce sex reversal even if 17α-methyltestosterone is the most common (Pandian and Varadaraj, 1990) for *Oreochromis mossambicus*; 17α-ethynyltestosterone (Shelton et al., 1981) with *O. aureus*; 17α-methylan-drostendiol (Varadaraj and Pandian (1987) with *O. mossambicus*; mibolerone (Torrans et al., 1988) with *O. aureus*; norethisterone acetate (Pandian and Varadaraj, 1990) with *O. mossambicus*; fluoxymesterone with *O. niloticus* (Phelps et al., 1992); trenbolone acetate with *O. aureus* (Galvez et al., 1996). Production of male tilapia through the use of androgens is very effective. Sex reversed "male" reached similar average weights as genetically male tilapia (Mair et al., 1995) and it does not require that a portion of the production be discarded as in manual selection, or that two separate stocks of fish be maintained as in hybridization (Phelps and Popma, 2000). The presence of hormone residue in adult fish has not yet been studied, thus its effect on consumers is not yet known and so his use is restricted. Hormones may also be difficult to obtain in some countries and hatchery facilities and skilled labour is required (Fortes, 2005).

Sex reversal by oral administration of feed incorporated with methyl testosterone is probably the most effective and practical method for the production of all male Tilapia, However, the technique has some limitations such as the uniform age of fish that should be used at the first feeding stage to ensure high reversal rate and less control of reversal efficiency especially when done in the natural environment where natural feed is present. Moreover, widespread use of large quantities of sex reversal hormones in hatcheries may pose a health risk to workers (Mair, 1997; López et al., 2007). This technique has achieved successful results up to 100% and feed with the male hormone is commercially available or can be prepared. One of its disadvantages though is the possibility of contaminating the water through wastewater due to non-consumed feed.

Another variant of the oral administration is the use of live bait that has been raised in an artificial environment enriched with male steroids. This technique has been used with Nile Tilapia fry and has obtained levels of masculinisation up to 99% (Contreras-Sánchez et al., 2004). Sex reversal by the immersion technique is achieved by immersing the eggs in different concen-trations of 17-αmethyl testosterone exposed for different times. The mechanism of action of the immersion technique is that the hormone is absorbed through passive diffusion across the lipid membrane of the eggs. During the embryonic development, gonadal differen-tiation can be affected by the administration of steroid sex hormone (Jobling, 1995) in the holding water. Strussman and Nakamura (2003) pointed out that the mechanism of action of exogenous steroids during sex differentiation is not sufficiently clear. Cagauan et al. (2004) evaluated sex reversal of Nile Tilapia *O. niloticus* by immersing the eggs in different concentrations of 17-αmethyl testosterone. Highest percent male of 91% was attained at 800 µg L-1hormone concentration at 96-h immersion time comparable with the 88 to 89% in 400 to 600 µg L-1hormone concentration at the same immersion time. Sex reversal by egg immersion may lessen the duration of treatment and lower the cost of hormone used relative to the traditional technique of sex reversal by oral admini-stration. However, this technique presents conflicting results possibly due to the rapid early development that limits the window of opportunity (Contreras-Sánchez, 2001) and these results are lower than those obtained with the immersion of fry (Gale et al., 1996; Fitzpatrick et al., 1999; López et al., 2007) and with the use of feed hormone (Manosrioi et al., 2004; Jiménez and Arredondo, 2000; Torres and Marquez, 2006).

Similarly to egg immersion, Gale et al. (1996) demonstrated that fry immersion for just three hours in 17α-methyldihydrotestosterone on two days resulted in masculinisation of Nile Tilapia with a success rate of 93%. It is therefore important to consider the number, time and duration of the immersion. Fitzpatrick et al. (1999) reported 90% of males obtained using treatments with 0.5 mg/L methyl testosterone in two immersions, with duration of 2 h each one, between day 10 and 13 after fertilization. López et al. (2007) also obtained 92.6% of males

using 1.8 mg methyl testosterone/L, with an immersion of 4 h, between 10 and 14 days after fertilization. This alternative technique of administering the sex reversal hormone may be of great help in hatcheries employing artificial incubation because of the greater control of sex reversal and lower risk to health of workers. On the other hand, these results are worse than using hormone on feed and this could yield to an uncontrolled reproduction with all the problems earlier mentioned.

GENETIC SEX DETERMINATION

The study of the influence of gene expression in performance traits like growth rate, feed conversion efficiency, body conformation, disease resistance and sex determination is an opportunity to meet the demands of fish production while ensuring profitability (Liu, 2007). Genetics is a field of study in constant development. Functional genomics is an emerging discipline that studies the effects of gene expression, genomic controls and transcriptional profiles and it is being applied to cultured fish species. The challenge is to determine exactly what each gene does in terms of the development and physiological functioning of the organism (Murphy, 2002).

A specific trait is the result of many genes working together and some genes involved in sex determination have already been found. Cyp19 is a gene that encodes P450 aromatase, the key enzyme catalyzing the conversion of androgens into estrogens (Tong and Chung, 2003). Estrogens play a crucial role in ovarian differentiation of non-mammalian vertebrates, including fish. There are results that suggest that Foxl2 affects the ovarian differentiation of the Nile tilapia by regulating aromatase expression and possibly the entire steroidogenic pathway. Foxl2 and Cyp19a1 are co-localized spatially and temporally in the female making them the earliest known markers for ovarian sex differentiation. Foxl2 can be considered as the pro-ovary, but anti-testis gene because the disruption of Foxl2 could stimulate the XX tilapia to reverse its sex from female to male partially or completely (Wang et al., 2008). Ijiri et al. (2007) studied the gonadal expression of 17 genes thought to be associated with gonadal sex differentiation in vertebrates, confirmed the role of FoxI2 and Cyp19a1a in ovarian differentiation and concluded that DMRT1 have a crucial role in testicular differentiation.

Kobayashi et al. (2008) also examined the expression profiles of tDMRT1 and Sox9a during gonadal sex differentiation and hormone-induced sex reversal. This study indicated that tDMRT1 is expressed in the germ-cell-surrounding cells and medullary-cell-mass cells, which differentiate into the efferent duct during testicular differentiation, irrespective of the genetic sex and suggest that Sox9a is not involved in the differentiation of the intratesticular efferent duct. They concluded that DMRT1 is a superior molecular marker for somatic testicular differentiation, and that DMRT1 and Sox9a play different roles in the testicular differentiation of tilapia. In addition, Wang et al. (2010) clarified the role of DMRT1 in their study by showing that it suppresses the female pathway by repressing aromatase gene transcription and estrogen production in the gonads of tilapia and possibly other vertebrates. Although, presently, it is not technically feasible to produce all male populations of tilapia by suppressing or activating genes, the knowledge needed to do this is being developed. Kuramochi et al. (2011) induced male-specific nest-building behavior in 70% of females treated with 11-ketotestosterone (11-KT) or methyl testosterone. This treatment increased the number of gonadotropin-releasing hormone type III (GnRH3) neurons, which presents sexual dimorphism in females to a level similar to that in males. These results indicate androgen-dependent regulation of GnRH3 neurons and nest-building behavior, suggesting that GnRH3 is importantly involved in sex reversal of male-specific reproductive behavior. However, the endocrine mechanism underlying sex reversal of reproductive behaviours remains unsolved.

CONCLUSIONS

In Tilapia culture, the desired goal efficiency rate for each technique to produce male Tilapia is between 98 and 100%. The technique employed in the majority of the developing countries is sex reversal because of its easy employment and high rate of success. Among the different techniques used for sex reversal (oral administration, egg immersion and fry immersion) the one that shows the best results is the fry immersion, with a higher success rate and without the risk to employees due to contact with the hormone during the preparation of the feed. Some other advantages of these techniques are: it takes less period of time, less water quality and quantity is needed, less hormone consumption and no influences from fish feeding behavior. Due to various environmental issues related to hormone use such as the possible effects of treatment residues on water quality and biodiversity, the countries with legislation against the use of hormones tend to use nonhazardous, consumer and environment-friendly methods to obtain all-male populations through genetic control. These methods, even if approved, are out of the common aqua farmer's technical capabilities.

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Full Length Research Paper

Morphotypes vis-a-vis genetic parameters of *Catla catla* (Ham.) and *Labeo rohita* (Ham.) backcrosses

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Backcross generations of *Catla catla* (Ham.) and *Labeo rohita* (Ham.) were developed in Central Agricultural Research Institute, Port Blair, South Andaman, India, using the technique of induced breeding for Indian Major Carps. The trend of morphometry through generation mean analysis indicates reduction of head size with respect to standard length, which is considered as a reduction of bone size within whole body biomass. The segregation pattern of dominant head morphometries of rohu and partial dominance of body morphometries of catla was supported by subsequent genetic evaluation through karyotyping, biochemical analysis and PCR-random amplified polymorphic DNA (RAPD) based molecular marker analysis indicating more genetic proximity of rohu with backcrosses than catla. The present study is significant for carp genetics with special reference to catla and rohu.

Key words: Backcross, catla, esterase, karyomorphology, molecular marker, morphometries, rohu.

INTRODUCTION

The two Indian major carps namely:- *Catla catla* (Ham.) and *Labeo rohita* (Ham.) are scattered naturally in various river systems of India, Pakistan, Burma and Bangladesh (Jhingran and Pullin, 1985). They are among the world's principal aquaculture species in terms of production (Hulata, 2001) and differ in many ways with respect to their phenotypic traits. As per the study of Basavaraju et al. (1995), larger head per unit body weight of catla is considered as a major disadvantage for freshwater aquaculture when the edible flesh content per unit body mass is concerned. Therefore, a good amalgamation of deep catla type body and narrow rohu type head is always a notion of considerable importance for aquaculture requiring apt hybridization.

Keeping this in view, the present study was undertaken to develop different backcross generations of catla and rohu with an aim to develop a new variety/strain with certain desirable traits. The desirable traits with aquaculture importance include narrower head, broader, longer and thicker body in percentage of total length and stouter caudal peduncle keeping in view of the percentage of edible flesh content in comparison with the total body weight. Success to achieve this target could be anticipated through selective breeding to make narrower and longer head, as well as, broader and thicker body by introgression of responsible gene(s). In similar genetic improvement programme, disease resistance, field performance and breeding capacity etc are also other parameters of concern but, those were not taken in to consideration in this study.

The major objective in the present study was to get different generations of Indian major carps namely:- *Catla catla* and *Labeo rohita* through systematic breeding and as it is very difficult to get parental generations, F1 and

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F2 hybrids, as well as, various backcross generation at a time due to their long generation period of 2 to 3 years. At the same time genetic analysis through evaluation of the developed progenies was done involving generation mean analysis (GMA) of morphometric parameters to get a clear trend followed by comparative karyotyping; esterase profiling and PCR-RAPD based molecular markers analysis.

Comparative karyotyping was essential to find any major change in the genome at chromosomal level as chromosomes are the simplest indicators of change in genome at cellular level through change in ploidy or any other change in the number of each type of chromosome, that is, metacentric, sub-metacentric, telocentric, sub-telocentric anatenctric, acrocentric, etc. The technique of biochemical genetics through isozyme analysis and PCR-RAPD marker based analysis was adopted to find the correlation between genetic distances/similarities between various generations of carps with respect to segregation of phenotypic trends from parents to progenies. This was desired to find genetic proximity of catla or rohu parents with other successive generations indicating the contribution towards development of longer and narrower head like rohu and broader, deeper and thicker body like catla.

MATERIALS AND METHODS

Developing the breeds of backcross generations

For the above purpose, the base stocks of parental generations were developed from the seeds of *Catla catla* (Ham.) and *Labeo rohita* (Ham.) in farm facilities of Central Agricultural Research Institute (CARI), Port Blair, Andaman, India (Tripathy et al., 2010) from the founder stocks of the parental generations developed from the seeds procured from the hatchery unit of Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, Odisha during 1987 as a part of hatchery development programme in CARI. During subsequent years, pure stocks of catla and rohu were developed through regular breeding programme of the institute to meet the demand of local farmers each year. Side by side, various tissue samples were collected and preserved for use in future; as well as, morphomtric measurements were documented for analysis. The mating design mostly followed was 1:1 brooders for each experimental analysis purpose whereas it was 2:3 or 1:3 (male:female) for farm requirement purpose. The breeds were maintained in separate pools and ponds without allowing any mix up.

The F_1 hybrids were developed by crossing catla and rohu and were designated as C×R or CR. Subsequently, the F_2 hybrids were produced from *inter se* breeding of F_1 progenies (CR×CR), the first backcross generation or B_1 was produced from F_1 and catla (CR×C), whereas B_2 from F_1 and rohu (CR×R). Hybridization of B_1 and rohu resulted in B_1R (CR×C)×R and BC_1F_2 were from *inter se* breeding of B_1 (CR×C) × (CR×C). A long and tedious process of breeding, hatchery management and maintaining the breeds in isolated ponds was undertaken from 1987 to 2008 to set the objective of backcross breeding and genetic evaluation taking the help of Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, Odisha, India and CARI from time to time. However the detail of induce breeding for the period of F_1, F_2 and backcross development is presented in Table 4.

Morphometry and generation mean analysis (GMA)

All metrical values of the morphometric parameters were converted to percentage of standard length and mean percentage values of each generation along with their standard error of means as analyzed Balon (1995). For the Generation Mean Analysis (GMA), three replication means of each morphometric ratio of all generations with corresponding values of variance and variance of means were used by GENRES. For this study, 73 catla, 50 rohu, 36 F_1 hybrids, 30 B_1 backcross, 30 F_2 hybrids, 30 B_2 backcross, 30 B_1R and 30 BC_1F_2 backcross were measured for various morphomteric parameters.

The age group of catla and rohu ranged from 1 to 3 years while those of F_1 and B_1 were between 6 months to 2 years. For the rest other generations, the age group varied between 6 months to 1 year. Various morphometric parameters included mean percentage values of head, trunk and tail length as well as their depth and thickness in percentage of standard length running from tip of the snout to the base of the caudal fin. For measuring each morphemetric parameter, replica of three set individuals were collected during harvest/sampling consisting of 25 to 30 each for catla and rohu. Those of other generations consist of 10 to 15 per sampling. The morphometric analysis mostly included mean percentage values of nine important parameters in percentage of standard length for length, depth and thickness of head, body and caudal peduncle; as well as, those of snout length, eye diameter and pre-dorsal, pre-pectoral, pre-anal and pre-pelvic length. The detail of morphometric parameters recorded is presented in Table 5.

The generation mean analysis was performed using the statistical software GENRES (1994) by putting the mean percentage values of morphometric parameter like length, depth and thickness of head and body for comparison. The data in Tables 1 and 2 present different scaled values as per scaling scaling and six parameter test, respectively and different inter-genetic interactions resulting in form of various epistatic phenomena, that is, duplicate or complementary epistasis. The scaling scaling test used means, variance and means of variance giving result in form of scaled values such as A, B, C and D scales with their corresponding variances and t values as per Haymann and Mather (1955). In these classical genetic model like scaling scaling test and six parameter model the co-efficient of variations which are the percentage values of standard deviations (Falconer, 1981; Mather and Jinks, 1982; Phanse and Sukhatme, 1995; Falconer and Mackay, 1996) are employed to get the final out put in form of all the four scales (A, B, C and D) and the genetic interactions values like M, D, H, I, j and L.

The scaled value like A is calculated by the software based on statistical formula, which is $A= 2B_1-P_1-F_1$ and variance of A (V_A)= $4V(B_1) + V(P_1)+ V(F_1)$ where B_1 indicates the mean values of the character of the 1st backcross generation, P_1 presents that of 1st parent (catla) and F_1 presents that of 1st filial progenies. The corresponding values of variances for each generation are represented by V (X_n). Similarly, the scaled value $B = 2B_2-P_2-F_1$ and $VB= 4V(B_2) +V(P_2) +V(F_1)$. The scaled value $C= 4F_2-2F_1-P_1-P_2$ and $Vc = 16 V(F_2) + 4V(F_1) + V(P_1) + V(P_2)$ and $D = 2F_2- B_1-B_2$ and $VD= 4V(F_2) + V(B_1) + V(B_2)$. The values of standard error for each scale is calculated by taking the square root of corresponding variances and t values mentioned in Table 1 are the proportion of each scale with respect to their standard errors having an exclusion limit of 1.96 at P =0.05.

The Table 2 presents another classical genetic test; that is, six parameter model of GMA to reveal non-interacting crosses giving means, variance and t values for mean effect (m), additive effect (d), dominance effect (h), additive × additive interactions (i), additive × dominance interactions (j) and dominance x dominance type interactions (l). The soft ware calculated the above parameters based on formulae:

(m) = mean genetic effect = that of F_2 generation for a morphometric measurement, (d) = Additive effect for a particular morphometric mean percentage value of all generations = the difference of B_1 and B_2 (B_1-B_2).

Table 1. Different scaled values of A. B, C and D with corresponding SE, variance and t values as per scaling scaling test of GMA.

Mean morphometric ratios in percentage of standard length	A ± SE: variance (t)	B ± SE: variance (t)	C ± SE: variance (t)	D ± SE: Variance (t)
Head length	-6.41± 2.71: 7.39 (-2.36)*	1.28 ±2.39: 5.73 (0.53)	3.15±3.33: 11.09 (0.94)	4.14±2.16: 4.67 (1.91)
Head depth	-4.98±1.98: 3.93 (-2.51)*	-3.76±1.23: 1.52 (-3.05)*	-6.24±4.33: 18.83 (-1.43)	1.25±2.31: 5.35 (0.54)
Head thickness	-1.24±1.83: 3.34 (-0.67)	-7.39±2.11: 4.46 (-3.49)*	-10.73±3.13: 9.85 (-3.41)*	-1.05±1.98: 3.92 (-0.53)
Body length	10.11±3.89: 15.19 (2.59)*	-1.23±2.13: 4.54 (-0.57)	-16.32±5.42: 29.42 (-3.01)*	-12.6±3.37: 1.39(3.73)*
Body depth	-5.74±4.38: 19.20 (-1.30)	-10.44±1.48: 2.2 (-7.03)*	-34.18±2.9: 8.40 (-11.78)*	-9.0±2.56: 6.569 (3.51)*
Body thickness	0.67±1.52: 2.31 (0.44)	-4.07±1.42: 2.03 (-2.85)*	-17.8±2.55: 6.52 (-6.97)*	-7.2±1.54: 2.38 (-4.66)*

**A, B, C and D: Different scales of genetic interactions, values in parentheses are the t values, * Significant at P, 0.05; A, $2B_1 – P_1-F_1$ and V_A, 4V (B_1) +V (P_1) +V (F_1) where $B_{1:}$ indicates the mean value of the character of the 1st backcross generation $P_{1:}$ presents the value of 1st parent (catla) $F_{1:}$ presents that of F_1 hybrids V (Xn): corresponding values of variances B= $2B_2-P_2-F_1$, V_B=4V (B_2) +V (P_2)+V (F_1); C=$4F_2-2F_1-P_1-P_2$, V_C = 16V (F_2)+4V(F_2)+V (P_1)+V (P_2) D = $2F_2-B_1-B_2$, V_D = 4V(F_2)+V (B_1)+V(B_2).

Table 2. Values of genetic interactions namely,- m, d, h, i, j and l ± standard deviation and type of epistasis.

Ratios	m	d	h	i	j	l	Type of Epistasis
Head length	37.96±4.37*	4.18±0.65*	-0.93±11.50	-8.28±4.32*	-3.84±1.76	13.41±7.0	Duplicate
Head depth	25.86±4.64*	4.26±0.39*	0.84±10.41	2.5±4.62	0.61±1.10	11.24±5.99	Complementary
Head thickness	16.57±3.89*	0.47±0.45	4.21±9.94	2.1±3.96	3.07±1.38*	6.53±6.11	Complementary
Body length	45.23±6.77*	-3.97±0.48*	58.28±16.60*	25.2±6.75*	5.67±2.19*	34.08±10.11*	Complementary
Body depth	14.36±5.15*	3.75±0.51*	20.91±14.38	18.0±5.12*	2.35±2.28	-1.82±9.37	Duplicate
Body thickness	3.43±3.10	-0.87±0.31*	25.14±7.60*	14.4±3.08*	2.37±1.02*	11.0±4.64*	Complementary

*** m: mean genic effect (F_2); V (m) = V $F_{2:}$ d: additive effect (B_1-B_2); V (d) = V B_1 + V $B_{2:}$ h: dominance effect (F_1- $4F_2$- ½ P_1 -½ P_2 + $2B_1$ +$2B_2$); V (h) = V F_1 + 16 V F_2 + ¼V P_1 + ¼V P_2 + 4VB_1 + 4V$B_{2:}$ i: additive x additive interactions ($2B_1$ +$2B_2$ -4 F_2); V (i) = 4V(B_1)+ ¼V(B_2)+ 16V(F_2); j: additive x dominance interactions (B_1- ½ P_1 -B_2 + ½ P_2); V(j) = V(B_1) +¼V(P_1)+ V(B_2)+ ¼V(P_2); l: dominance x dominance interactions (P_1+ P_2+ $2F_1$+4 F_2- $4B_1$-$4B_2$); V(l) =V(P_1)+ V(P_2)+ 4V(F_1)+16V(F_2) +16V(B_2)+ 16V(B_1); Significant at P = 0.05.

The value of h (dominance effect) was calculated by the mean percentage values of F_1-$4F_2$-1/2P_1-1/2P_2+$2B_1$+$2B_2$. Their corresponding variances were calculated as V (m) = V (F_2), V (d) = V (B_1) + V (B_2), and V (h) = V (F_1) + 16 V (F_2) + ¼ V (P_1) + ¼ V (P_2) + 4 V (B_1) +4 V (B_2). The values for all the three genetic interactions are: (i) = $2B_1$+$2B_2$ -4 F_2 and V (i) = 4 V (B_1) +¼ V (B_2) + 16 V (F_2). Similarly, (j) = B_1-1/2 (P_1)-B_2+ ½ (P_2) and V (j) = V (B_1) + ¼ V (P_1) + V (B_2) + ¼ V (P_2) and (l) = P_1 + P_2 + $2F_1$ + $4F_2$-$4B_1$-$4B_2$ and V (l) = V (P_1) + V (P_2) + 4 V (F_1) + 16 V (F_2) + 16 V (B_2) + 16 V (B_1).

Karyomorphology

After acclimatization, the individuals were subjected to starvation for 24 h in separate aquarium and were injected with Concanavalin-A (1mg/ml in Phosphate Buffer Saline PBS of pH 7.4 @ 1ml/100 g body weight prior to 48 h of dissection). Second dose was administered 24 h prior to actual sacrifice. Then they were injected with colchicine @ 1ml/100 g body weight 2 to 3 h prior to dissection. Kligermann and Blooms (1977) method was adopted followed by flame drying and stained in 4% Giemsa working solution (BDH). Counting of chromosome number and assignment of karyomorphologies to each set of chromosome was done following the study of Levan et al. (1964).

Esterase profiling

According to the study of Abersold et al. (1987), Esterase (EC: 3.1.1.) was profiled by vertical slab gel (5% native polyacrylamide gel electrophoresis) in discontinuous buffer system (Rechardson et al., 1986) using Bio-Rad made mini electrophoretic apparatus (Mini Protein-II, Catalogue no 165) at 4°C with running voltage of 250 V for 2 h. The co-dominant isozyme marker bands were assigned different codes for different loci like aa, AA, BB and bb for homozygous genotype and AB Ab, aB, bB, ab, Aa for heterozygous genotypes. The data based on isozyme polymorphism of esterase markers were analyzed and the genetic distance matrix was constructed by GENEPOP-3.2.

PCR-RAPD generated Molecular Marker analysis

Extraction of genomic DNA was done by Sambrook et al. (1989) with suitable modification (Barman et al., 2003), following the routine protocol of phenol-chloroform- isoamyl alcohol. Polymerase chain (PCR) reactions were set up in sterile environment by 5 pmol of selected primers (oligonucleotide decamers) and 0.5 U of Taq DNA to amplify in thermal cycler Gene Amp 9700 (Applied Biosystems). Initial denaturation at 94°C (4.0 min) was followed by 35 cycles in 1.0 min (94°C), 1.0 min (36°C) and 2.0 min (72°C). Final extension for 7.0 min at 72°C was allowed. The experiments were conducted for 10 sets decamer random primers. The amplified DNA was subjected to 1.8 % agarose gel electrophoresis along with 100 bp DNA ladder or gene ruler (FERMENTAS: MBI #SM0321) and were visualized under UV light using gel documentation system (Uvi tech, Techne, UK). Band scoring was done as 0 for absence or 1 for presence of bands by Quantity One (BIO-RAD) and was analyzed by computer-simulated software POPGENE 32 for construction of dendrogram based on genetic distance matrices according to the study of Nei (1972, 1978) by Unweighted Paired Group Method with Arithmetic Averages (UPGMA).

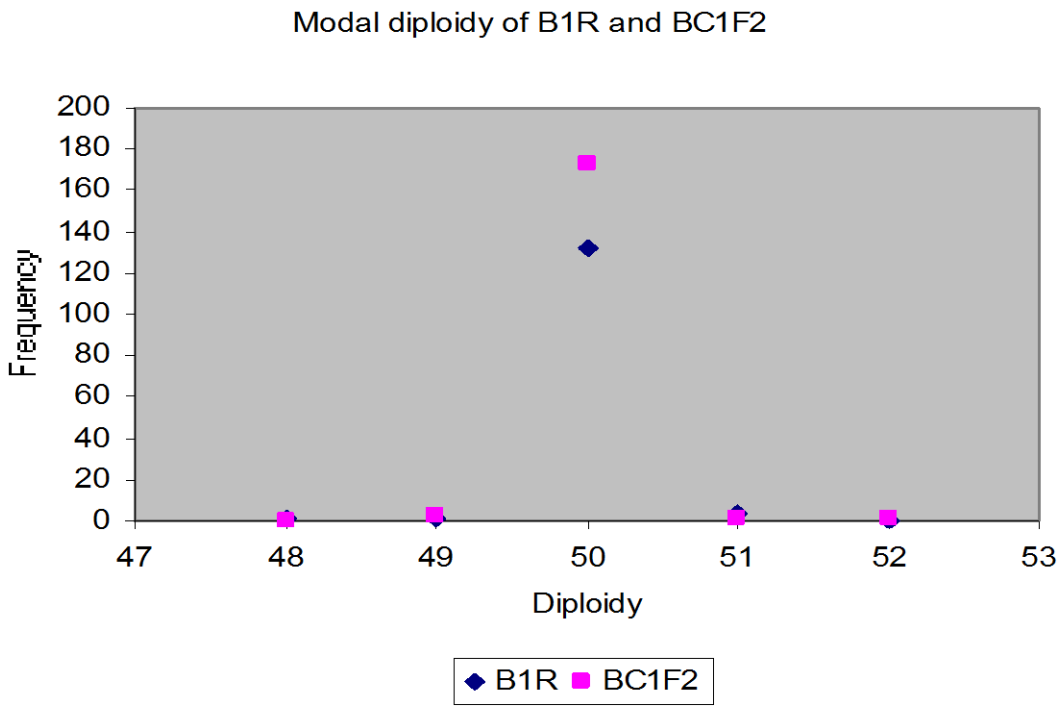

Figure 1. Modal diploidy (2n = 50) in backcrosses of catla and rohu.

Table3. Comparative karyomorphologies of backcrosses and parental carps.

Carp	Chromosomal morphologies						Reference
	2n	FN	M	Sm	St	T/A	
Catla	50	78	12	16	-	22	Zhang and Reddy (1991)
Rohu	50	-	6	16	8	20	Nagpure (1997)
F_1	50	88	12	10	16	12	Jana (1993)
B_1R	50	-	14	10	10	16	*
BC_1F_2	50	-	8	12	22	8	*

******** FN, Fundamental number; M, metacentric; Sm, sub-metacentric; St, sub telocentric; T/A, telocentric/anacentric; ***,** As per the present findings; Sample size (n), B_1R (15) and BC_1F_2 (17).

The soft ware POPGENE (Yeh et al., 1999) calculated genetic distances and identities based on the formulae of pair-wise similarities (S_{AB}) as per Lynch (1990) originally based on Nei (1972) and Nei (1978) for similarities index, using the data from polymorphic primers where $S_{AB} = 2\ N_{AB}/(N_A+N_B)$, where N_{AB} is the number of DNA fragments between individuals A and B, and N_A and N_B are the total number of fragments possessed by individuals A and B. The mean pair-wise similarity S was computed as $S = \Sigma S_{AB}/n$ where it is the arithmetic mean of all S values. As per Lynch (1990), the variance of S was calculated as V (S) = 2S (1-S)(2-S)/ N(4-S). N stands for average number of DNA fragments per individual.

RESULTS

Morphometry and generation mean analysis

Six mean morphometric proportions namely:- length, depth and thickness of head and body were analyzed in backcrosses. The head morphometries were observed intermediate to parental catla and rohu but akin towards rohu, whereas body morphometries were more similar towards catla. The B_1 progenies showed four characters that is, length and thickness of head as well as length and depth of body more similar to parental rohu where as B_2 backcrosses showed four ratios namely, length and depth of head, as well as, length and thickness of body of rohu. The B_1R backcrosses showed head thickness like catla where as body depth like rohu and BC_1F_2 showed two characters like depth and thickness of head similar to rohu. The mean head length in percentage of standard length for parental rohu in current study was found similar to those in earlier observations of Chondar (1985), whereas it was highest for catla and lowest for rohu.

As per the scaling scaling test (Table 1) of generation mean analysis, the generation means for head length from

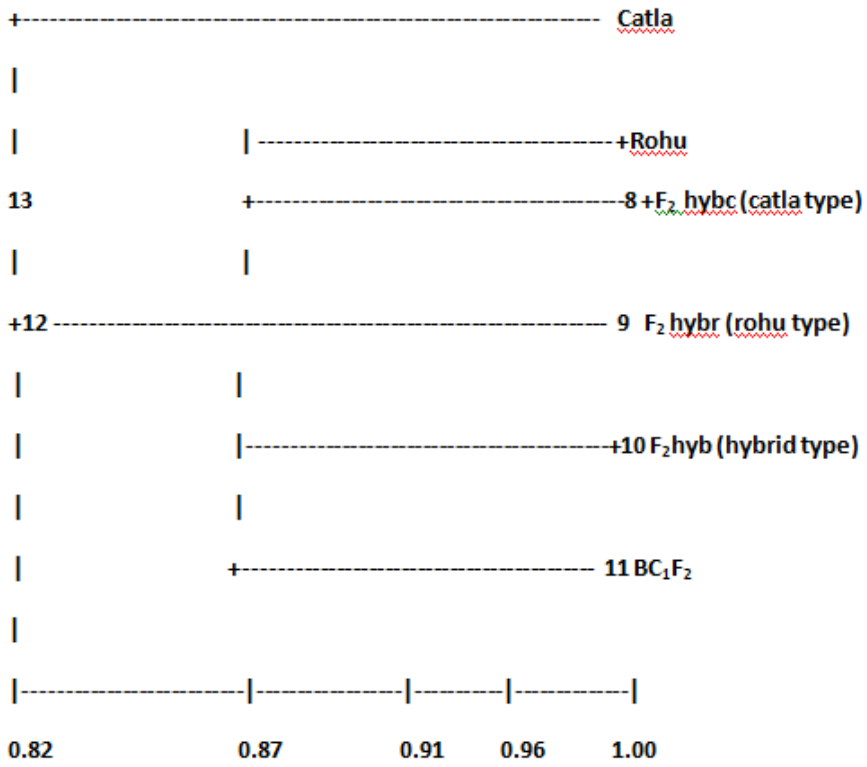

Figure 2: Dendrogram based on esterase profiling.

all generations showed the only significant value of scale A whereas generation mean morphometries for depth, scale A and B were found significant. Scales B and C differed significantly for thickness. Out of all the four scaled values for body length, only B was found non-significant and other three scaled values were significant. For body depth parameters in all generations, three scales namely, B, C and D differed significantly. Body thickness showed the only non-significant scaled value of A.

For the six parameter model presented in (Table 2), head length resulted in three significant parameters namely:- mean effect (m); additive effect (d) and additive x dominance epistasis (i) but that for the head depth, only m and d were significant. Similarly for head thickness, the significant parameters were mean genic effect (m) and additive x dominance interactions (j). All parameters were found significant for body length where as body depth showed significant parameters of m, d and i. All parameters for body thickness were significant except the mean genic effect (m).

Karyomorphologies

The backcross progenies in the present finding confirm same diploidy as earlier observation in catla, rohu and F_1 (Figure 1 and Table 3). The number of metacentric chromosomes is 14 in B_1R and 8 in BC_1F_2. Similarly, the number of sub-metacentric in B_1R is 10 and 12 in BC_1F_2. Number of sub-telocentric chromosomes in B_1R and BC_1F_2 are 10 and 22. The number of telocentric/acrocentric chromosomes is 8 in BC_1F_2 and 16 in B_1R.

Esterase profile

There exists positive correlation for genetic distance between both the backcross generations and B_1R backcrosses were more distantly correlated to rohu then BC_1F_2 (Figure 2). Highest value of genetic distance of rohu was observed with catla followed by BC_1F_2, F_2 and B_1R. B_1R backcrosses showed maximum distance with catla and minimum with F_2. The BC_1F_2 generation showed maximum genetic distance with catla followed by rohu, B_1R and F_2.

PCR-RAPD based molecular markers

A total of 105 (one hundred and five) bands in all generations were analyzed where an average of 6 to 8 bands per primer was amplified. Primers like OPY-7 and OPY-12 amplified highest number of DNA fragments. The size range of amplified DNA fragments was 3000 to 300 bp. Almost 35.23 % of the total amplified bands were either catla or rohu specific and only 40 to 100% of them were polymorphic. Catla was found having maximum genetic identity with B_1 and minimum with F_1. That in ascending order was with rohu, BC_1F_2, B_2, B_1R and F_2.

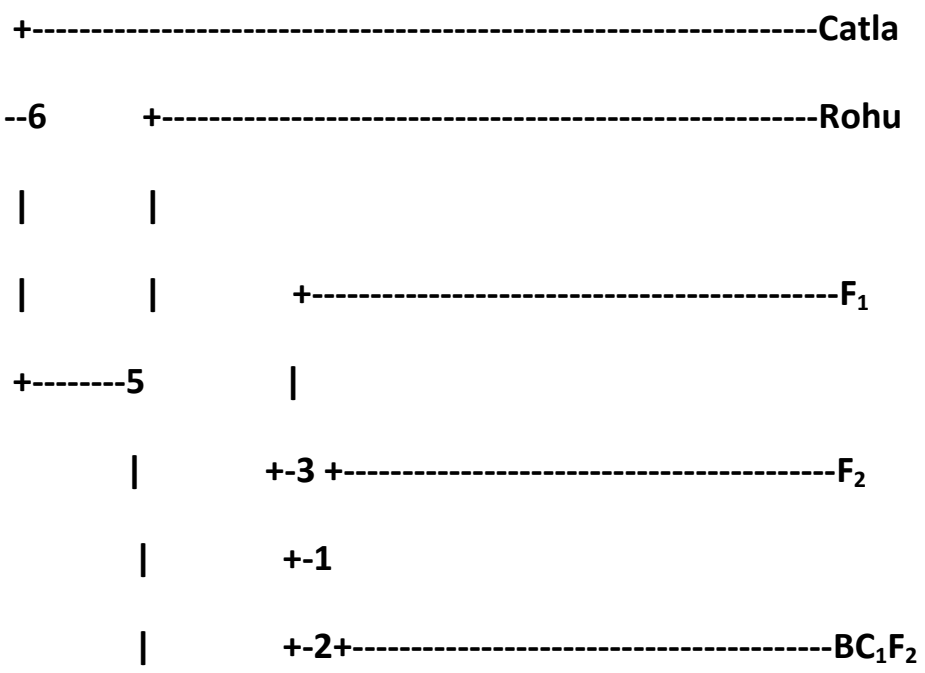

Figure 3. Dendrogram based on dominant PCR-RAPD markers.

Similarly, the genetic identity of rohu with carps in ascen-ding order was with catla, B_1, B_2, B_1R, F_2, BC_1F_2 and F_1 respectively and those of B_1R in ascending order was with catla, B_1, rohu, B_2 and BC_1F_2 respectively (Figure 3). Finally, the genetic identity of BC_1F_2 in ascending order was catla, B_1, rohu, F_2, F_1, B_2 and B_1R.

DISCUSSION

Selection in backcross programmes is used to either improve the genetic value of plant and animal populations or fine map quantitative trait loci. Both cases are helpful in understanding of the genetic bases of quantitative traits variation. Development of backcross progenies in Indian major carps that is, catlta, rohu and mrigal is not a well adopted practice as they have long generation cycle of three years approximately. But similar attempts were made in some other carps and non-carps earlier (Beherends et al., 1988; Jayaprakash et al., 1988; Andersons and Collins, 1995; Galbreath and Thorgaard, 1995) for various purposes of aquaculture in general; as well as, to understand their genetics. The present study was meant to gain inside to the carp genetics with respect to catla and rohu, as they contribute a lion share to the Indian market of freshwater captive fisheries. Backcrossing is a well-known and long established breeding scheme where a characteristic is introgressed from a donor parent into the genomic background of a recurrent parent (Hospital, 2005). Backcrossing is also useful to dissect the genetic architecture of quantitative traits because it isolates a gene, or chromosomal region, in a different genetic background (the genetic background of the recurrent parent). In fact, it is one of the few reliable methods to validate the additive effect of a quantitative trait locus (QTL) or a candidate gene. The experiments were conducted to introgress rohu characteristics with narrower head to hybrids and backcrosses.

Overall behavior of B_1 backcrosses in the present study was similar to those of catla being easy to be caught in net but those of other backcrosses were closer to rohu. The mean head length in percentage of standard length for parental rohu in current study was found similar to those in earlier observations of Chondar (1985), where as it was highest for catla and lowest for rohu. The values of head depth in percentage of standard length were observed highest for catla and lowest for rohu so also the same for body length in percentage of standard length Body depth in percentage of standard length in the present study were lower than those reported earlier (Chondar, 1985). As per Falconer and Mackay (1996), the scaling scaling test informs that significance of any of the four scales that is, A, B, C or D indicates the presence of epistasis and the type of epistasis is revealed by the significance of specific scaled values. Significance of A and B scales indicate presence of all the three types of genetic interactions such as:

i. additive × additive,
j. additive × dominance and
l. dominance × dominance interactions.

Similarly, significance of scaled value C suggested dominance × dominance interactions (j) and that of D scale value indicates additive × additive genetic interaction (i). Significance of both C and D scaled values indicate pre-

Table 4. Detail of induced breeding of various generations of carps.

Hormones				Egg release (Lt)	Weight(kg)	Spawn recovery(thousand)	Carps
♀	♂	♀	♂				
1	2	3	4	5	6	7	8
C	R	PGE	PGE	11.0	0.4	5.0	F_1
C	R	PGE	PGE	20.0	2.4	180.0	F_1
CR	CR	OVP	OVP	5.0	0.2	1.0	F_2
CR	C	PGE	PGE	4.5	0.3	5.0	B_1
CR	C	PGE	PGE	8.0	0.6	12.0	B_1
C	R	PGE	OVT	18.0	2.1	135.0	F_1
CR	CR	PGE	PGE	28.0	2.6	460.0	F_2
CR	R	PGE	OVT	7.0	0.35	6.0	B_2
CR	R	PGE	PGE	21.0	1.30	46.0	B_2
CR	C	PGE	PGE	12.0	0.95	33.0	B_1
CR	CR	PGE	OVP	24.0	2.2	*	F_2
CR	R	PGE	PGE	21.0	1.30	53.0	B_2
CRXC	R	OVP	PGE	7.0	1.5	50.0	B_1R
CRXC	CRXC	PGE	PGE	10.0	1.2	180.0	BC_1F_2
CRXC	CRXC	PGE	PGE	14.0	1.8	225.0	BC_1F_2
CRXC	R	PGE	PGE	5.0	0.2	13.0	B_1R
CRXC	R	OVP	PGE	6.0	1.0	40.0	B_1R
CRXC	CRXC	PGE	PGE	4.0	0.8	45.0	BC_1F_2

C, catla; R, Rohu; CR, hybrid of catla female and rohu male; CRxC, B_1 backcross generation developed by crossing CR hybrid female and catla male; F_2, developed by crossing CR hybrid *inter se* breeding; B_2, developed by CR hybrid female x rohu male; B_1R, developed by crossing B_1 backcross female and rohu male; BC_1F_2, developed by crossing B_1 backcross female and male (*inter se* breeding); PGE, Pituitary gland extract; OVP, ovaprime; OVT, ovatide.

sence of both (i) and (j) interactions. In the present study it was found that significance of all four scaled values indicated the presence of all the three types of genetic interactions (i, j and l) for overall expression of head length in percentage of standard length in all the studied generations of catla, rohu and backcrosses. Same was the result for head depth in percentage of standard length, indicating complicated polygenic interactions. Similar result for head thickness in percentage of standard length due to complicated polygenic interactions indicated to give more emphasis on dominance × dominance interactions (i) while considering for selective breeding. This required more attention towards out breeding to obtain the desired percentage of head thickness in various backcross generations of catla and rohu. While considering the body morphometries as per the scaling scaling test of GMA

Table 5. Measurements of morphometric parameters in mean percentage of standard length (mm) with values in parentheses indicating Standard Error of Mean (SEM).

Ratio	P_1	P_2	F_1	F_2	B_1	B_2	B_1R	BC_1F_2
				In % of standard length				
Head Length	35.42 (0.39)	26.0 (0.6)	32.5 (32.1)	32.116 (0.5)	28.103 (0.04)	27.916 (0.72)	31.084 (0.27)	30.859 (0.33)
Head Depth	26.29 (0.04)	19.8 (0.3)	25.9 (0.26)	21.633 (0.9)	22.62 (0.5)	20.533 (0.43)	23.252 (0.18)	21.65 (0.47)
Head Thickness	18.85 (0.25)	15.6 (0.6)	18.52 (0.2)	16.9 (0.044)	16.16 (0.3)	16.17 (0.492)	18.935 (0.24)	16.15 (0.316)
Body Length	65.33 (0.89)	73.9 (0.6)	66.55 (0.6)	67.2 (0.60)	72.43 (1.0)	72.096 (0.69)	70.18 (0.429)	70.147 (0.58)
Body Depth	33.68 (0.67)	26.9 (0.5)	29.72 (0.4)	24.766 (0.8)	28.70 (1.0)	25.73 (0.499)	26.083 (0.22)	25.652 (0.47)
Body Thickness	17.30 (0.30)	17.0 (0.3)	16.1 (0.25)	13.23 (0.30)	18.18 (0.4)	15.9 (0.37)	15.072 (0.24)	17.24 (0.465)
C.P. Length	16.968 (0.2)	16.5 (0.1)	16.55 (0.0)	18.633 (0.0)	17.48 (0.3)	15.48 (0.21)	15.8 (0.06)	20.40 (2.505)
C.P. Depth	9.614 (0.33)	12.4 (0.1)	13.05 (0.0)	11.46 (0.16)	11.9 (0.09)	11.83 (0.049)	11.77 (0.041)	13.01 (0.107)
C.P. Thickness	9.958 (0.19)	6.7 (0.05)	8.13 (0.02)	7.16 (0.096)	7.91 (0.06)	6.45 (0.061)	7.844 (0.047)	9.11 (0.221)
Snout Length	9.76 (0.061)	9.1 (0.06)	10.5 (0.04)	9.91 (0.13)	9.86 (0.06)	10.116 (0.11)	8.8 (0.016)	11.394 (0.08)
Eye Diameter	6.78 (0.091)	6.01 (0.2)	7.72 (0.09)	6.56 (0.068)	7.17 (0.14)	8.183 (0.19)	7.261 (0.013)	8.494 (0.054)
Pre-Dorsal	49.21 (0.16)	46.9 (0.4)	49.77 (0.1)	46.53 (0.55)	46.4 (0.43)	47.25 (0.66)	48.97 (0.109)	46.521 (0.19)
Pre-Pectoral	30.93 (0.74)	25.5 0.4)	29.3 (0.18)	28.03 (0.33)	27.9 (0.27)	28.2 (0.34)	28.25 (0.10)	28.868 (0.19)
Pre-Pelvic	54.968 (1.3)	51.8 (0.2)	53.59 (0.2)	45.7 (0.932)	51.76 (0.2)	52.4 (0.47)	52.85 (0.42)	47.62 (0.674)
Pre-Anal	76.09 (1.63)	76.63 (1.25)	77.70 (0.2)	71.83 (2.4)	71.68 (1.3)	77.466 (1.59)	77.038(0.29)	73.536 (0.26)

P_1, Catla; P_2, Rohu; CP, Caudal Peduncle.

indicated significant values of B,C and D scales only for head depth in percentage of standard length, indicating the presence of additive x additive (i) and dominance × dominance interactions only. For body length and thickness in percentage of standard length all types interactions (i, j and l) were indicated by the scaling scaling test.

In the six parameter test model of GMA, the values of h and i having similar signs (+ ve or – ve) indicate presence of complementary epistasis and those with opposite signs indicate duplicate epistasis. According to the study of Viana (2000), the coefficient of the component (i) is same as the additive component (d) and the coefficient of the

Table 6. Genomic contribution from parents to offspring in various carp generations.

Carp Generation	Maternal Parent	Designation of the carp	Catla Genome (%)	Rohu Genome (%)
Catla	Catla	P_1	100	0
Rohu	Rohu	P_2	0	100
F_1 Hybrid	Catla	CxR or CR	50	50
F_2 Hybrid	F_1	CR x CR	50	50
B_1	F_1	CR x C	66	33
B_2	F_1	CR x R	33	66
B_1R	B_1	CRC x R	50	50
BC_1F_2	B_1	CRC x CRC	66	33

component (j) is same as the dominant component (h) in any generation.

The probable reason for shifting of head morphometry towards rohu type producing backcrosses with rohu like head (narrower) can be attributed to a probability of masking genes for head morphometry in rohu genome though dosage wise higher from rohu parents only in parental rohu and B_2 backcross only (Table 6). This is true when such morphometric characters are not monogenic but polygenic trait with complicated genetic interactions. For head morphometry, involvement of more than one gene present in rohu genotype to make the head narrower and less deeper contributing more for the expression in subsequent generations of catla and rohu. But for body morphometry which is another polygenic trait, the rohu genome itself is not getting fully expressed and the catla genome masking partially.

It is also apparent in Table 6 that contribution of parental catla genome as a whole is more in case of parental catla (100%), B_1 (66%) and BC_1F_2 (66 %) but higher whole genome contribution with parental rohu alike in cases of rohu (100%) and B_2 (66%) which is not showing any clear trend to confirm more genetic proximity of rohu with other subsequent generations. But, considering the contribution of maternal genome with rohu linked genes, it is significant that rohu alike genome present to some degree in maternal parents of rohu and also in cases of production of F_2, B_1 and B_2 where F_1 is the maternal parent and F_1 itself is having genetic dose from rohu parents. Similarly, for the development of B_1R and BC_1F_2 generations, the maternal parents are B_1 backcrosses with rohu genome's contribution. This might have induced the rohu genome to mask the catla genome for development of head morphometry but not for body morphometry.

Hybrids between Atlantic salmon (*Salmo salar*, At) and brown trout (*Salmo trutta*, Bn) were highly viable and expected to be functionally sterile due to major interspecific karyotypic differences (Galbreath and Thorgaard, 1995). In contrast to this in the present study, intergeneric differences of catla (genus Catla) and rohu (genus Labeo) has not produced similar results but all the hybrids and backcross generations developed were highly viable and fertile due to chromosomal compatibility with same diploid number. The karyotype of B_1R and BC_1F_2 progenies in the present findings show 2n=50 with differences in their karyomorphologies. The rough estimation of metacentric and submetacentric chromosome (M + Sm) in catla is 28 and in rohu 22. Similarly, estimation of sub-telocentric and telocentric chromosome (St + T) which looks similar and difficult to identify is 22 in catla and 28 in rohu. Both comparative estimations are just opposite to each other in catla and rohu parents. Keeping these facts in view, the (M+Sm) value in all generations are 22, 24 and 20 in F_1, B_1R and BC_1F_2 generations alike rohu. So also the values of St + T are 28, 26 and 30 in F_1, B_1R and BC_1F_2 generations alike rohu. Although these are very crude estimations not to be ascertained with full conformity but points more towards genetic proximity with rohu parents. However, stronger and more authentic comparison application of chromosomal banding techniques could have served better. But as a first hand or preliminary information at chromosomal level of investigation through karyotyping it can be accepted that, the genomic organizations of different carp generations are in more proximity with rohu genome.

The dendrogram for esterase profile based on genetic distance matrix showed two distinct clusters of carps linked together where one with rohu and excluding catla keeping aside in a separate branch. From this observation it was clear that, rohu parents are more close to all the subsequent generations of carps rather than the catla parents. Hence, all generations of carps were found more linked to rohu than catla. In the phylogenetic tree based on PCR-RAPD markers, two distinct groups of carps were observed where rohu was found along with all carps except catla keeping aside in a separate branch, similar to earlier observations as per esterase marker. The overall genetic evaluation showed more proximity of backcrosses with rohu rather than with catla. A possible explanation ascribed is due to disruption of co-adapted gene complexes or alteration in dominance relationships between alleles. Such disruption might be brought by the differences for the alterations in various components of development of hybrids and backcrosses as compared to parental stock that is, catla and rohu. For head morphometries it is desirable and must be welcomed in future perspectives for freshwater aquaculture in Indian peninsula.

A correlation test among genetic distance matrices of molecular and biochemical marker showed a coefficient of 64%. The success of breeding the backcrosses and their genetic evaluation to find out the heredity may be attributed to nature itself, which accepted intra-generic hybridization resulting in viable hybrids due to their compatibilities at chromosomal level. The dominance of rohu head morphotypes over catla was considered to be of high significance from aquaculture point of view. The trend of morphometric evaluation is appropriately supported by findings from isozyme and molecular marker analysis. The type of inheritance exhibited by these backcrosses of catla and rohu has not been reported before but appears similar to that reported in backcrosses of other vertebrate species. These results show the importance of backcrossing as well as use of genetic markers to determine parental contributions and gene segregation in species hybrids.

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Genetic differentiation of watermelon landraces in Mozambique using microsatellite markers

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Landraces of seed and dessert type watermelons from three provinces of Mozambique with distinct agro-ecological conditions, including humid and semi-dry areas from the north to the south of the country, were analysed to assess their genetic differentiation. Ninety-six accessions (269 plants) were tested with 24 microsatellite markers resulting in 110 alleles. Analysis of molecular variance showed that 63% of the total variation in the plant material could be explained among the accessions, while 37% of the variation was within accessions. Molecular variance between material used for seed extraction and dessert consumption explained 34% of the total variation whereas villages explained 27% of the molecular variation among the sampled locations. Structure analysis revealed that the material could be differentiated into three genetic groups. The seed types clustered in one genetic group, irrespective of provincial origin. Dessert type accessions from the semi-arid south and the central part were assigned to a second genetic group, while accessions from the northern more humid region of the country were assigned to a third genetic group. The observed genetic diversity may reflect farmer selection under different agro-ecological conditions or an introduction of material from different sources into the growing areas.

Key words: *Citrullus lanatus*, DNA fingerprinting, landraces, Mozambique, microsatellites, simple sequence repeats (SSR).

INTRODUCTION

Watermelon [*Citrullus lanatus* (Thunb.) Matsun and Nakai] is an annual species, including cultivated, semi-domesticated and wild forms, which are widely distributed in tropical and sub-tropical areas (Robinson and Decker-Walters, 1997; Jeffrey, 2001). Three subspecies of *C. lanatus* are recognised by Jeffrey (2001): subsp. *vulgaris* (Schrad. Ex Eckl. et Zeyh.) Fursa, subsp. *lanatus* including var. *citroides* (L. H. Bailey) Mansf. Ex Grebensc, and sub sp. *mucosospermus* (Fursa), encompassing the 'egusi' seed watermelons. However, in more recent literature, the species has been differentiated into two botanical varieties: *C. lanatus* var. *lanatus* and *C. lanatus* var. *citroides* (Jarret et al., 1997; Dane and Liu, 2007; GRIN, 2012). Using this classification, the dessert watermelon and the 'egusi watermelons', belong to var. *lanatus,* whereas the wild forms (*citroides* group, also called citron melons) from the Kalahari Desert ('tsama') whose rind may be used for preserves, jellies and conserves, are assigned to

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Abbreviations: SSR, Simple sequence repeats; **RAPD,** random amplified polymorphic DNA; **CTAB,** cetyl-trimethyl-ammonium-bromide; **PCR,** polymerase chain reaction; **PIC,** polymorphic information content; **MDS,** multi-dimensional scaling.

Table 1. Use type and regional origin of 96 water-melon landrace accessions from Mozambique included in the present SSR study.

Use	Province			Total
	Cabo Delgado	Manica	Gaza	
Dessert	25	27	38	90
Seed	3	0	3	6
Total	28	27	41	96

var. *citroides* (Dane and Liu, 2007). The seeds and flesh of *citroides* may also be used for food preparations (Maggs-Kölling and Christiansen, 2003; Dane and Liu, 2007), but are often maintained for animal feed (cow-melons) (Mujaju et al., 2011).

Watermelon is known for its use as a dessert 'fruit', but has other versatile uses in Africa. In Southern Africa, farmers cultivate dessert, seed and cooking type watermelons (Maggs-Kölling et al., 2000; Mujaju et al., 2011). The fruits are nutritionally important as a natural source of citrulline and carotenoids such as lycopene and β-carotene, a precursor of vitamin A (Setiawan et al., 2001; Edwards et al., 2003; Collins et al., 2007). As a potential drought tolerant plant (Kawasaki et al., 2000; Yokota et al., 2002), it is of particular interest in semi-arid areas of Africa. Under such conditions, it is mostly intercropped with cereals to reduce the risk of complete crop failure and thereby ensuring food security (Munisse et al., 2012).

Knowledge on the genetic diversity and differentiation of watermelon resources is useful to develop and implement effective strategies for conservation and sustainable use. Information generated by genetic diversity and differentiation studies is a step towards useful identification of optimal parental combinations in plant breeding, assessment of the degree of variability for classification of accessions, the development of core collections as well as assessment of the extent of genetic erosion in genebank collections (Mohammadi and Prasanna, 2003). Since it is virtually impractical to characterise the whole genome, the assessment of crop diversity depends on the availability of suitable genetic markers. DNA based molecular markers are environmentally neutral and effective in providing useful information required for assessment of genetic variation (Kalia et al., 2011). One of the most useful and robust DNA based molecular marker systems are the microsatellites or simple sequence repeats (SSRs) which are short tandem repeats of one to six DNA nucleotides distributed throughout most plant genomes (Wang et al., 1994; Li et al., 2002). The difference in number of repeats in microsatellite regions can generate high levels of polymorphism, and are useful for a variety of genetic studies including the assessment of relationship between organisms; variety discrimination/genetic finger printing, genetic mapping and marker assisted breeding (Kalia et al., 2011). Several authors have evaluated SSRs for their potential use to assess relationships among *Citrullus* accessions or to discriminate varieties (Jarret et al., 1997; Guerra-Sanz, 2002; Joobeur et al., 2006; Levi et al., 2006). Based on SSR data, Jarret et al. (1997) differentiated *Citrullus* accessions into 4 major groups, namely *C. lanatus* var. *lanatus, C. lanatus* var. *citroides*, hybrid *C. lanatus* var. *lanatus* x *C. lanatus* var. *citroides* and *C. colocynthis*. While these studies have contributed to our understanding of the phenetic classification within the genus *Citrullus*, a low level of genetic diversity was found in material of cul-tivated watermelon. Nevertheless, 'egusi' seed type and dessert type watermelon accessions from Mali have been differentiated genetically (Nantoumé et al. 2013).

There is still little information on watermelon landrace diversity from Southern Africa. In Namibia, Maggs-Kölling and Christiansen (2003) validated the farmer classification of cultivated watermelon into dessert, seed and cooking types based on morphological cluster analysis. A diversity study using SSR and random amplified polymorphic DNA (RAPD) markers on landraces of cow-melons (*C. lanatus* var. *citroides*) and edible landraces from Botswana, Namibia, South Africa, Zambia and Zimbabwe revealed a genetic differentiation between the two types (Mujaju et al., 2011).

In view of developing national and regional strategies for conservation and sustainable use of watermelon genetic resources, it is of interest to broaden the knowledge related to the diversity of landraces in other countries of Southern Africa. This study aimed at assessing the differentiation and genetic structure of dessert and seed type watermelon landraces collected from farmers' fields in Mozambique.

MATERIALS AND METHODS

Plant material

Plant material for this study consisted of 96 landrace accessions collected from farmers providing a handful of seeds (100-200 seeds) in 12 villages in Cabo Delgado (Northern Mozambique), 8 villages in Manica (Central Mozambique) and 12 villages in Gaza (Southern Mozambique) in 2008. The accessions included seed and dessert types (Table 1) which are further described by Munisse et al. (2011). Among the dessert type watermelons, farmers recognized white, light red, and red fleshed types. The geographical coordinates for the accessions were recorded using a hand held global GPS positioning system (eTrex Venture HC, Garmin). Seeds of the commercial dessert cultivar type Sugar Baby, obtained from two different seed companies (Harris Moran Seed Company, Davis, USA, and Weibull Seed, Sweden), were included as controls.

DNA extraction

Seeds were germinated in plastic pots filled with sand in a greenhouse at 30-33°C. Total genomic DNA was extracted from young leaves of one to eight single watermelon plants randomly selected per accession (269 plants were sampled). From each plant, six leaf discs were sampled, using the lid of a 1.5 ml Eppendorf tube, and collected in 1.2 ml collection tubes (Qiagen). The leaf samples were freeze-dried for 24 h. The material in the tubes was subsequently crushed to a fine powder using glass beads on a mixer mill (Retsch, Type MM301) using a frequency of 30/s for 2 min, then the samples

Table 2. SSR's amplified from genomic DNA from 96 watermelon landraces from Mozambique, number of alleles and polymorphic information content values.

SSR	Number of allele	PIC[a]	SSR	Number of allele	PIC
ASUW13[b]	4	0.02	MCPI-25[c]	5	0.18
Cgb5009[b]	3	0.27	MCPI-26[c]	5	0.46
CLG8288[b]	4	0.38	MCPI-27[c]	4	0.39
MCPI-03[c]	5	0.43	MCPI-28[c]	5	0.53
MCPI-07[c]	2	0.31	MCPI-32[c]	3	0.50
MCPI-09[c]	7	0.63	MCPI-33[c]	7	0.28
MCPI-10[c]	3	0.34	MCPI-34[c]	3	0.49
MCPI-12[c]	7	0.49	MCPI-37[c]	5	0.16
MCPI-13[c]	10	0.74	MCPI-39[c]	4	0.42
MCPI-14[c]	6	0.12	MCpI-42[c]	6	0.40
MCPI-20[c]	5	0.18	MCPI-44[c]	1	0.00
MCPI-21[c]	4	0.24	MCPI-47[c]	2	0.14
Average				4.7	0.34

[a],According to Botstein et al., 1980; [b], Levi et al., 2006; [c], Joobeur et al., 2006.

were rotated and the milling was repeated. DNA was extracted using a modified cetyl-trimethyl-ammonium-bromide (CTAB) method (Saghai-Maroof et al., 1984). The modifications included isoamyl-alcohol substitution for octanol, the final rinsing step used 70% ethanol, and DNA was dissolved in 1 x TE buffer. The protocol was adjusted to enable extractions in collection microtubes (Qiagen). DNA concentrations were adjusted to 20 ng/µl based on spectro-photometer measurements (GeneQuant *pro,* Amersham Pharmacia Biotech).

Polymerase chain reaction (PCR) amplification and allele scoring

The PCR amplifications were performed in 96 well plates (96 Multiply, Sarsted) in a PCR GeneAmp 2700 thermal cycler (Applied Biosystems) using 24 selected SSR primers developed by Levi et al. (2006) and Joobeur et al. (2006), listed in Table 2. PCR for generation of SSR fragments used the M13-tail nested PCR approach with fluorescent labelling of the products according to Schuelke (2000). Forward primers were 5'-tailed with a 19-base pair M13 universal sequence: 5´- CACGACGTTGTAAAACGAC-X-3´, where X denotes the specific microsatellite primer sequence. Each PCR reaction of 7.5 µl contained 60 ng genomic template DNA, 1x PCR reaction buffer (Ammonium buffer, GenScript), 0.2 mM of each of the four dNTPs (Bioline Ltd.), 7.5 µg bovine serum albumin BSA (Calbiochem), 0.6 mM $MgCl_2$, 0.4 pmol forward primer with 5'-M13 tail, 1.6 pmol reverse primer, 1.6 pmol fluorescently labelled M13 primer (labelled with 6-FAM (blue), NED (green) or VIC (yellow) fluorescence (Applied Biosystems), and 0.2 U Taq DNA polymerase (GenScript).

The thermal cycler was programmed as follows: 1 initial cycle at 94°C for 1 min; 30 cycles of denaturation at 94°C for 1 min, primer specific annealing at 50-64°C for 1 min and extension at 72°C for 1 min; followed by 8 cycles of denaturation at 94°C for 1 min, annealing at 55°C and extension at 72°C for 1 min, and a final extension step at 72°C for 5 min; finally held at 4°C. Amplified PCR products labelled with the three different fluorescent dyes were pooled and a ROX labelled (6-carbon-X-rhodamine) molecular size standard (13 fragments, 58-508 bp) was added before denaturation at 95°C for 2 min. SSR fragments were separated by capillary electrophoresis on an AB 3130*xl*Genetic Analyzer (Applied Biosystems and Hitachi Ltd.). Fragments were sized relative to the internal ROX size standard and scored with manual bin setting using the software program GeneMarker, version 1.75 (SoftGenetics LLC & Bioké). Control samples of DNA from cultivar Sugar Baby were included in all 96 well plates.

Data analyses

Genetic analyses were performed with macros in Excel (Microsoft Excel v. 2007, Redmond, USA). Polymorphic information content (PIC) values were calculated as in Botstein et al. (1980), and genetic distances were calculated using modified Rogers's distance (Wright, 1978). The matrix of genetic distances was used in non-parametric multi-dimensional scaling (MDS) with the R Statistics Package (R Development Core Team, 2008) with the MASS package (Venables and Ripley, 2002). The GenAlEx vers. 6.4 software program (Peakall and Smouse, 2006) was used as an add-in in Excel to determine the proportions of the molecular variance (AMOVA) among and within the accessions, and to estimate partitioning of variation among accessions, use groups and villages, using 9999 permutations. A matrix of geographical distances was calculated on the basis of longitude and latitude GPS positions from sampling sites to perform a Mantel test (Mantel, 1967) in GenAlEx, to study correlation between this matrix and the genetic distance matrix using 9999 permutations.

A model-based approach using a Bayesian algorithm, implemented in the software package STRUCTURE version 2.3.3 (Pritchard et al., 2000) was used to explore and identify population clusters. This analysis included samples of the commercial cultivar Sugar Baby. Overall, the model assigns individual genotypes to unknown clusters (groups) by considering the presence of Hardy-Weinberg equilibrium, so that population groups as far as possible are not in disequilibrium (Pritchard et al., 2000). STRUCTURE was run with number of clusters (K) from 2 to 11. Each K was run 20 times with a 'burn-in period' of 10.000 rounds, assuming an admixture model. The most likely number of genetic groups was estimated by the statistical ad hoc criterion (ΔK) described by Evanno et al. (2005). For an assessment of a likely assignment of landrace types to a proposed grouping in 3 clusters, the average proportion of the individuals, belonging to seed and dessert types at the village level, was calculated (q-value).

Table 3. Analysis and partitioning of molecular variance for SSRs among watermelons from Mozambique for accession, use group, province and village level.

Source of variation	df	MS	% Var.	PhiPT[a]	P-value
Among accessions	95		63	0.626	<0.001
Within accessions	265		37		
Total[b]	360				
Between use groups	1		34	0.383	<0.001
Within use groups	267		66		
Total	268				
Among provinces	2		8	0.076	<0.001
Within provinces	266		92		
Total	268				
Among villages	31		27	0.268	<0.001
Within villages	241		73		
Total[c]	271				

AMOVA, 96 accessions included a total sample number of 269; [a]PhiPT, test statistics. P-value based on 9999 permutations; [b] 24 accessions were only represented by one plant, and 22 accessions were only represented by two plants. For the analysis, the data from these accessions were duplicated. [c]One village was represented by only one accession, and one by only two accessions. For the analysis, data from these were duplicated.

RESULTS

Marker statistics

The 24 primer pairs produced an array of 110 different alleles in the tested landraces, with an average of 4.7 alleles per SSR marker locus and a maximum number of 10 alleles recorded for MPCI-13 (Table 2). The PIC varied from 0.0 (SSR marker MCPI-44 was monomorphic) to 0.74 (SSR marker MPCI-13) with a mean of 0.34 (Table 2). Markers MPCI-13, MPCI-9, and MPCI-28 were the most informative with PIC values of 0.74, 0.63 and 0.53, respectively.

Analyses of molecular variation

Analysis of molecular variance based on SSRs revealed that 63% of the variation could be explained among accessions and 37% of the variation within accessions (Table 3). A separate AMOVA showed that 34% of the variation could be explained by usage (dessert or seed). Variation among provinces accounted for only 8% of total variation while the variation among districts explained 13% of the total variation (results not shown). When analysing the variation at the village level, 27% of the variation was accounted for by the villages. The Mantel test for collection distances of sample material up to 1573 km showed a low positive significant correlation between genetic and geographical distance ($R^2 = 0.035$, $P<0.001$).

Genetic structure

STRUCTURE analysis showed the highest likelihood for differentiation of the tested material into 3 genetic groups (Δ-K = 3.82) followed with lower likelihood for structuring into 10 groups (Δ-K = 1.10) (Figure 1). The three genetic groups are shown in Figure 2 using multiple dimensional scaling based on Rogers's modified distance. One of the groups (group 1) is separated from the other two, whereas genetic group 2 and 3 are close to each other and show some overlap (Figure 2). Further analyses were performed with special interest on the three group structure.

In Mozambique, the dessert landrace accessions generally have names associated with flesh colour: white, light red and red. However, colour categories did not associate with the genetic grouping of accessions (data not shown). The analysis of molecular variance indicated that use group (seed or dessert type) and village origin explained a considerable amount of the variation in the material (34 and 27%, respectively). Therefore, we attempted to explain the three genetic groups based on these two factors. Average q-values for the three genetic groups are presented for the use groups at the village level in Table 4.

Among the seed types in the study, one village from Cabo Delgado (Milamba) and two villages from Gaza (Nwamalanzdele and Muzamane) cultivated accessions, which exclusively belonged to genetic group 1. Another two villages (Nhanupo and Maputo) from Cabo Delgado cultivated seed types which were assigned partly to genetic group 1 and group 2 (Table 4). Thus overall, the seed type watermelons from Mozambique belong to genetic group 1 (5 out of 6 accessions), irrespective of province of origin, with genetic admixture to group 2 in the humid province of Cabo Delgado.

For the dessert type accessions, there are indications that genetic grouping relates to the province origin (Table

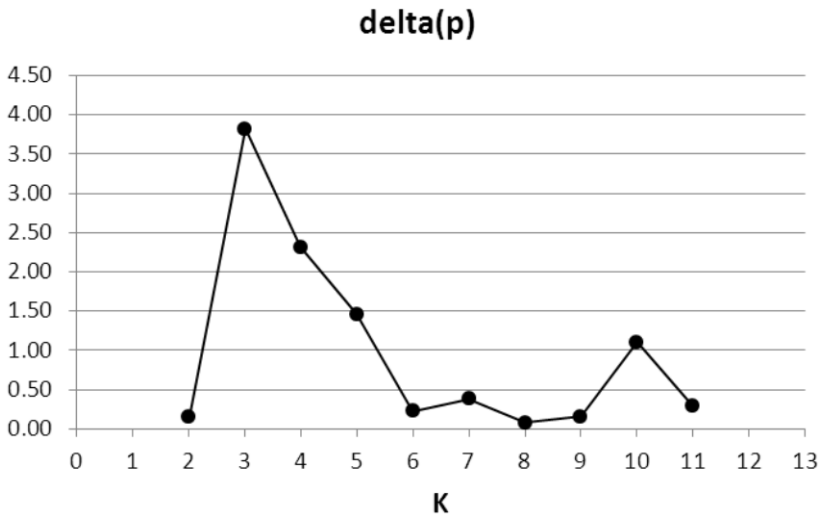

Figure 1. Delta(p) for number of groups in the watermelon material suggested by STRUCTURE analysis.

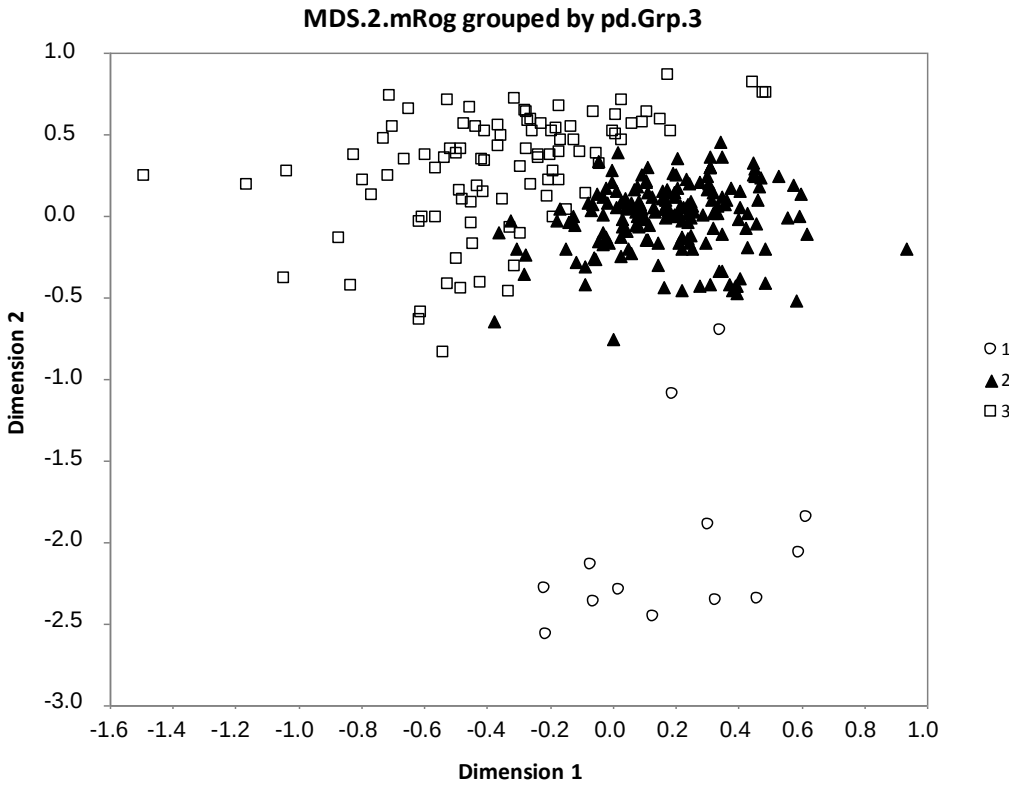

Figure 2. Plot of first two dimensions from multi-dimensional scaling based on Rogers's modified distance of 24 SSR loci with the three proposed structure groups.

4), although it was only explained by 8% of the variation in AMOVA. Villages from the northern Cabo Delgado province tended to grow dessert types with a genetic group 2 and 3 background. Five of the 10 villages in Cabo Delgado (Lianda, Macapa, Mpeme, Nhanupo and Nhanupo Bloco Ncunama) had material exclusively assigned to group 3, while another three villages (Mucojo, Lago Chico and Koko) had accessions assigned to both group 2 and group 3. The dessert accessions from the remaining two villages of Cabo Delgado (Cipembe

and Miula) were not assigned clearly to any of the three groups. Overall, the majority of dessert water-melon accessions from the north (16 out of 25 accessions) had a genetic background most similar to group 3.

Only dessert types were collected from villages in the central Manica province. Accessions from four of the eight villages sampled (16 de Junho, Guezane, Magagodza and Muda Serracao), were clearly assigned to group 2. Another two villages in Manica (Nhangary and Massimino) had dessert accessions assigned to group 3 and two villages (Benhenhe and Bunga) grew material which shared a genetic background with both group 2 and 3 (Table 4). Overall, the majority of dessert type accessions in the central province revealed a genetic background mainly belonging to group 2 (22 out of 27 accessions).

In the southern province of Gaza, dessert types mainly belonged to group 2, irrespective of the village. Thus, among the 11 villages of Gaza sampled during this study, seven villages (Machaila, Mapai, Mapunganhana Mavue, Namandjio, Nwamalandzele and Xifumana) grew dessert type watermelons, which were exclusively assigned to group 2 (24 accessions). However, accessions from four of the villages (Chituri, Madlambuti, Munginge and Mutchele) showed genetic admixture to group 1 and group 3 (q-value>0.2).

DISCUSSION

The average PIC value of 0.34 obtained in this study of 96 accessions from Mozambique and two commercial cultivars of Sugar Baby is somewhat lower than that reported in other watermelon diversity studies with material from southern Africa. Joobeur et al. (2006) reported a PIC value of 0.53 using 36 polymorphic SSR markers on watermelon material including 12 genotypes of *C. lanatus var lanatus* and *C. lanatus* var *citroides.* Studying 8 accessions belonging to the same two *C. lanatus* varieties, a PIC-value of 0.79 was obtained by Mujaju et al. (2010). Although such PIC estimates depend on both the diversity of the sampled material as well as the polymorphism of the SSR markers used for genetic analysis, there are some indications that these, mainly dessert type landraces grown in Mozambique, are not highly diverse. The two most informative markers in this study (MPCI-13 and MPCI-28) were also highly informative in the studies by Joobeur et al. (2006) and Mujaju et al. (2010). Average number of alleles per SSRs locus was 4.7 in this study, which is comparable to the average value reported by Mujaju et al. (2010) using SSR markers.

Several previous studies showed a difference in the genetic background between dessert type watermelons (*C. lanatus* var. *lanatus*) and semi cultivated seed types (*C. lanatus* var. *citroides*) (Jarret et al., 1997; Joobeur et al., 2006; Mujaju et al., 2010). In this study, AMOVA showed that 34% of the genetic variation was attributed to difference between the two types (seed and dessert). The seed types mainly belonged to one genetic group (group 1: 5 out of 6 seed type accessions) with some admixture to group 2 and the dessert types differentiated in two groups (group 2 and 3). Genetic admixture between the two main types (seed and dessert) has also been reported by Mujaju et al. (2010) for cow-melons in Zimbabwe. The morphology of the seed types is remarkably distinct from the sweet dessert types. For instance, the leaves of the seed types tend to have less lobes and a lower leaf blade ratio (length/width ratio) closer to 1 than the dessert types. Most of them also have firm flesh. Cross-compatibility between seed and dessert type water melons is expected, leading to introgression of genes from group 2 of the dessert type when insect pollination can take place and plants are not isolated by distance. Nevertheless, the present study supports the differentiation of seed and dessert type watermelons in Mozambique.

Regarding dessert landraces from Mozambique, it is generally difficult to assign them to distinct landraces as farmers generally give them different names associated with the colour of the flesh. In our study, flesh colour alone is not sufficient to differentiate landraces and this supports previous studies (Dane and Liu, 2007). Instead, there are some genetic distinctness between dessert type accessions from different regions of Mozambique. In the humid northern Cabo Delgado province, the majority of dessert watermelon accessions have a genetic background belonging to group 3. Interestingly, the two commercial Sugar Baby cultivars also belonged to this group, although most of the commercial production of watermelon takes place in the southern province of Gaza.

The majority of the dessert accessions in the central region of Manica belonged to the genetic group 2, and this is the case for all accessions from the southern dry province of Gaza. This substantiates the AMOVA result, showing that 8% of the variation in the material could be explained by the province. Interestingly, there seems to be a plausible explanation for the dessert group structure related to geography. Cabo Delgado in the north is isolated from the central province of Manica and southern province of Gaza. Also, Manica and Gaza are neighboring provinces with more similar agro-ecological zones, so trade and exchange of material more likely takes place here than between these two provinces and Cabo Delgado. This may explain why the dessert types in these two provinces are genetically similar (group 2) with some admixture to group 3. This admixture may be related to the introduced material from other regions with a genetic background similar to that of Sugar Baby.

Also, contributing to the provincial differences is the fact that external cultural influence has been quite different in the north and the central and southern part of Mozambique. Cabo Delgado in the north has substantial contact with ecological communities in Tanzania, while the southern region of Gaza is influenced more from the surrounding countries Zimbabwe and South Africa.

Cultivation practices for watermelon in semi-arid areas of Gaza are different from those in the humid Cabo

Table 4. Proportion of the genome (q-value) allocated to watermelon types and collection sites at the village level, in three genetic STRUCTURE groups.

Type	Village	Number of accession	Group 1 (q-value[a])	Group 2 (q-value)	Group 3 (q-value)	Group[b]
Province Cabo Delgado (North) with high rainfall						
Dessert						
	Lianda	1	0.118	0.007	**0.874**	**3**
	Macapa	2	0.002	0.021	**0.976**	**3**
	Mpeme	2	0.137	0.013	**0.851**	**3**
	Nhanupo[c]	1	0.002	0.015	**0.983**	**3**
	Nhanupo, Bloco Ncunama	2	0.027	0.014	**0.959**	**3**
	Mucojo	5	0.016	**0.674**	***0.311***	**2**, 3
	Lago Chico	1	0.003	**0.608**	***0.389***	**2**, 3
	Koko	8	0.045	***0.247***	**0.708**	2, **3**
	Chipembe	2	***0.333***	***0.409***	***0.258***	1, 2, 3
	Miula	1	***0.329***	***0.351***	***0.320***	1, 2, 3
Seed						
	Milamba	1	**0.981**	0.003	0.017	**1**
	Nhanupo[c]	1	**0.615**	***0.369***	0.017	**1**, 2
	Maputo	1	***0.222***	**0.777**	0.009	1, **2**
Province Manica (Central) with medium rainfall						
Dessert						
	16 de Junho	3	0.003	**0.921**	0.076	**2**
	Guezane	4	0.016	**0.889**	0.095	**2**
	Magagodza	2	0.003	**0.926**	0.072	**2**
	Muda Serracao	1	0.005	**0.914**	0.082	**2**
	Nhangary	1	0.002	0.020	**0.977**	**3**
	Massimino	4	0.004	0.110	**0.886**	**3**
	Benhenhe	2	0.006	**0.662**	***0.333***	**2**, 3
	Bunga	10	0.006	**0.615**	***0.379***	**2**, 3
Province Gaza (South), semi-arid						
Dessert						
	Machaila	3	0.002	**0.825**	0.172	**2**
	Mapai	4	0.004	**0.970**	0.026	**2**
	Mapunganhana	1	0.003	**0.991**	0.007	**2**
	Mavue	7	0.033	**0.851**	0.116	**2**
	Namandjio	4	0.010	**0.853**	0.138	**2**
	Nwamalandzele[c]	1	0.004	**0.960**	0.035	**2**
	Xifumana	4	0.016	**0.945**	0.039	**2**
	Chituri	3	***0.333***	**0.653**	0.014	1, **2**
	Madlambuti	5	0.105	**0.626**	***0.269***	**2**, 3
	Munginge	2	0.004	**0.797**	***0.200***	**2**, 3
	Mutchele	4	0.058	**0.609**	***0.333***	**2**, 3
Seed						
	Nwamalanzdele[c]	1	**0.989**	0.007	0.003	**1**
	Muzamane	2	**0.995**	0.003	0.002	**1**
	32 villages	**96**				
Dessert	Cv. Sugar Baby	1	0.003	0.025	**0.972**	**3**

[a],q-values: Boldface: q>0.6; *Boldface italic:* 0.2<q<0.6. [b]Group, Groups mentioned are those represented with q-values>0.2. Bold face is the dominating group. [c]From the villages Nhanupo and Nwamalanzdele was collected both a seed and a dessert type accession.

Delgado region. In contrast to areas with relatively high precipitation, the semi-arid areas like Gaza have a limited choice of crops that are drought tolerant. Watermelon production is one of few choices. Farmers in Gaza pay more attention to their watermelons. They sow their seeds in separate holes in the field and select for sweetness of the flesh, thinner rind and larger seeds. In contrast, in the more humid Cabo Delgado, most farmers sow watermelons by simple scattering of seeds on the soil, and they are not actively performing seed selection (Munisse et al., 2011).

The present study reveals a genetic structure in the cultivated watermelon types in Mozambique. The genetic differences associate to some extent with geographical separation, perhaps caused by different cultural influence and cultivation practices between the north and the central/south. The result is the development and perhaps introduction, of watermelons with distinct genetic backgrounds in the two regions of Mozambique. Further knowledge on the genetic diversity of the cultivated African watermelons may lead to higher awareness locally and globally of the considerable genetic resource available for the crop on the continent.

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Full Length Research Paper

Effects of different chemical materials and cultural methods on growth and yield of winter wheat

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To determine the effects of different chemical and cultural methods on the growth of winter wheat, six treatments were carried out: Conservational irrigation, non-irrigation, water absorbent polymers (WAP), liquid mulching film (LMF), water-saving irrigation (WSI) and subsoiling tillage (SST). The results show that winter wheat could use more water from soil profile though WAP, LMF and SST treatments; only LMF could use extra water for yield while both WAP and SST could not increase yield. SST could not increase yield of winter wheat. Both LMF and WAP treatments could help in maintaining leaf chlorophyll content and leaf water content which may help in maintaining photosynthetic ability in late growing periods. Furthermore, more dry matter partitioning to reproductive organs is observed in LMF and WAP treatments. LMF might be favorable for yield when grown under lower soil moisture conditions, while the application of WAP might not help in yield producing in field both in high or low soil moisture conditions. A reasonable irrigation quantity may be needed when applying WAP, while LMF could be used in any meteorological and/or soil water conditions.

Key words: Winter wheat, water absorbent polymers, liquid mulching film, subsoiling tillage

INTRODUCTION

Winter wheat (*Triticum aestivum* L.) is a main crop in Huang-Huai-Hai plain. Huang-Huai-Hai plain locates in north China and is one of the most important grain production regions in China (Zhang et al., 2005, 2006), which accounts for 53% of wheat production in China and about 15% of the total national grain production (Liu and Chen, 2005). Though the average annual precipitation in Huang-Huai-Hai plain is from 500 to 600 mm, the distribution in seasons is not even. More than 70% of the total precipitation was in the period from July to September. As a result, precipitation from October to next June (the winter wheat growing season) ranges only from 100 to 180 mm. About 25 to 40% water shortage for the requirement of wheat production (Zhang et al., 1999). Supplemental irrigation is required in winter wheat growing season. However, Huang-Huai-Hai plain has only 7.2% of the total national water resources, and no sufficient surface water resources are available for irrigation. Shortage of water resources has become the major limiting factor for wheat production (Liu et al., 2002; Zhang et al., 2005, 2006). Thus, it is necessary to adopt water-saving agriculture countermeasures to achieve the largest increase in water use efficiency of winter wheat (Li et al., 2007).

It is reported that in this area, about 60% in the early growth season and 30% in late growth season of total soil evapo-transpiration is from the soil evaporation (Kang et al., 1995; Xie, 1998; Liu et al., 2002; Zhang et al., 2004). Thus, to reduce soil evaporation is an important method to maintain soil moisture. Subsoiling tillage is used in field commonly for maintaining soil moisture (Zhang et al., 2007; Li et al., 2008a, b). In recent years, water absorbent polymers (WAP) were used widely in agriculture, especially in horticulture. Experiments were also done in

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Abbreviations: WAP, Water absorbent polymers; **LMF,** liquid mulching film; **SST,** subsoiling tillage; **WSI,** water-saving irrigation.

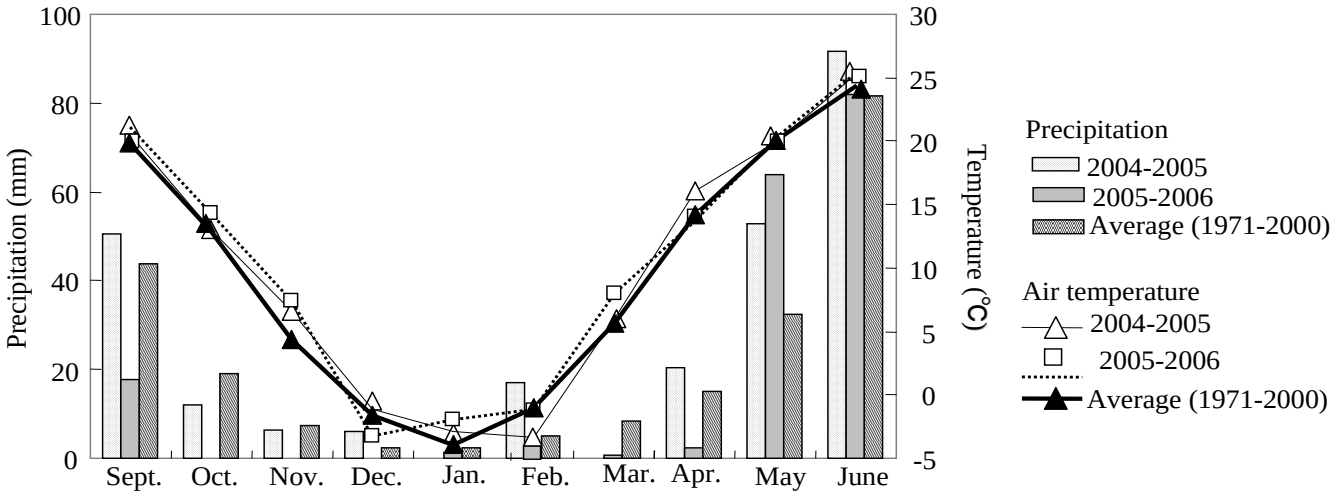

Figure 1. Monthly changes in precipitation and air temperature in Changping, Beijing in 2004–2006.

field crop. The results showed that WAP could improve the crop yield in maize, tomato, and so on (Yu et al., 2006; Zhang et al., 2007; Zhao et al., 2006; Yamane et al., 1990). WAP are less used in field crops not only because of its high price, but also because of its contention about soil physio-chemical properties and ecological environment as it is mainly made from chemical materials. In our alternate research, a new kind of material, liquid mulching film (LMF) has been synthe-sized with biological material which considered harmless to environment. LMF can be directly sprayed on soil surface and form a clear film on soil surface. It is reported that LMF could increase temperature, conserve soil moisture and improve the growth rate of plants (Yu et al., 2003; Zhang et al. 2003). Furthermore, LMF could ignore the plastic pollution and could also be used in the crop that plastic film could not be used, such as winter wheat (Huang et al., 2004; Zhang et al., 2005).

In this study, we intended to make a comparison among physical and chemical (SAP and LMF) methods applying on winter wheat. The objectives were: To determine the yield and yield performances of winter wheat when applied WAP and LMF; to find the effect of WAP and LMF on maintaining soil moisture and to discuss the differences in water efficiency of physical and chemical methods of different water-saving methods on soil moisture and yield.

MATERIALS AND METHODS

The experiments were done in the Changping experimental base (N 40.18°, E 116.23°) of the Chinese Academy of Agricultural Sciences in Beijing, China in winter wheat growth season from September 2004 to June 2005 and from September 2005 to June 2006. The local wheat cultivar (*T. aestivum* L. cv. CA0045) was used in this study. Twenty-four plots were used in this experiment. The area of each plot was 4 × 6 m. The sowing density was 225 kg ha^{-1} and the row distance was 23 cm in both years. Six methods (treatments) were carried out in this study. The treatments were: (1) Conservational irrigation (CI: Control, the same irrigation quantity as the commercial field around the base); (2) Non-irrigation (NI: only precipitation in growing period); (3) Water-saving irrigation (WSI: About 20% and 12.5% less than that of CI in 2004-2005 and 2005-2006, respectively); (4) Water absorbent polymers (WAP); (5) Liquid mulching film (LMF) and (6) subsoiling tillage (SST). The WAP (Bai Jinzi, Boya Co. Ltd., Tangshan, China) was put 20 cm depth under soil under the seedling row. The quantity was 60 kg hm^{-2}. LMF was sprayed equally on the soil surface after seedling. LMF was conducted in the Lab of agricultural disaster mitigation, Chinese Academy of Agricultural Sciences (CAAS). The irrigation quantities of WAP, LMF and SST were the same as that of WSI. Field was irrigated in winter-crossing stage, jointing stage and grain-filling stage. The soil moisture in 10 cm levels (1.4 m depth) was measured in week intervals with time-domain reflectometry (TDR). The actual quantum yield of PSII, the relative leaf water content, the relative values of leaf chlorophyll content were measured at jointing stage, heading stage and grain-filling stage in 2004-2005. The flag leaves were chosen for all above measurements. The actual quantum efficiency of PSII ($\Delta F/Fm'$) was measured using a pulse amplitude modulation chlorophyll fluorometer (PAM-2000, Walz, Effeltrich, Germany). Three leaves from each treatment and 5 places of each leaf were measured. Leaves from each treatment were taken and imme-diately sealed in plastic bags. The leaves were oven-dried for 24 h. The weights before and after drying were measured and the relative water content of each leaf was calculated. The relative values of leaf chlorophyll content were measured by SPAD-502 (Minolta, Japan). Five leaves were chosen for one species, and 5 places were measured in one leaf. The data were all averaged. The dry matter accumulation at different growing stages in 2004-2005, yield and yield components in harvest in both years were determined. The data were subjected to analyses of variance (ANOVA) to determine the effects of treatments, and the mean differences were adjudged by Duncan multiple range test (DMRT) at 0.05 probability level.

RESULTS

Meteorological and irrigation quantity

Figure 1 shows the meteorological condition in Changping meteorological station. The average monthly temperature varied a little among years. There is large

Table 1. Irrigation quantity of six treatments and precipitation in growth period (mm).

Treatment	2004-2005			2005-2006		
	Irrigation	Soil*	Total	Irrigation	Soil	Total
CI	225.0	-7.2	342.3	180.0	46.4	245.6
NI	0.0	32.0	156.5	0.0	88.1	107.3
WSI	180.0	-5.4	299.4	167.5	65.2	251.9
WAP	180.0	-7.3	297.2	167.5	84.4	271.1
LMF	180.0	-4.9	299.6	167.5	80.4	267.1
SST	180.0	-12.3	292.2	167.5	78.4	265.1
Precipitation		124.5**				19.2

*Means soil means the water consumption in soil profile from 0.140 cm, **means the precipitation in the growing season.

difference in precipitation within two growing seasons. It was 124.5 mm in 2004-2005 and 19.2 mm in 2005-2006, respectively.

Table 1 shows the irrigation quantity and total water consumption in both years. In 2004-2005, the total irrigation quantity of CI (Control) was 225 mm and the other treatments were 180 mm. Besides CI, the soil moisture increased at harvest time when compared with those before sowing. The total water consumption with the most in CI and the least in NI was 342.3 and 156.5 mm, respectively. The other treatments ranged from 290 to 300 mm. The total water consumption of NI was 45% that of CI, and other treatments were around 85 to 87% of CI, indicating that 180 mm was enough for wheat growth in 2004-2005. Thus, the irrigation quantity was adjusted to 180 mm for CI in 2005-2006, and the other treatments were 80% of CI, was 167.5 mm. The total water consumption was 245.6 and 107.3 mm for CI and NI, respectively. The other treatments ranged from 250 to 270 mm. The total water consumption of NI was 43% that of CI, and other treatments were around 100 to 110% of CI.

Eco-physiological parameters

Soil moisture

Figure 2 shows the changes of the water content at soil profile in different treatments with time. The water consumption was mainly concentrated in 0 to 50 cm depth. The difference among the treatments and years were not obvious. The soil water content in 0 to 60 cm depth was much higher in 2004-2005 as compared with that in 2005-2006. Oppositely, in 60 to 140 cm depth, the values was lower in 2004-2005 than that in 2005-2006, indicating that the winter wheat might abort more water from soil in upper profiles. The water content of SAP and LMF were higher than those of other treatments both in upper and lower profiles, indicating that both SAP and LMF might help in maintaining soil moisture, especially in deep soil profiles.

SPAD value, relative leaf water content and the actual quantum efficiency of PSII

The SPAD values in Flag leaf, the 2nd leaf and the 3rd leaf from top decrease as time especially in NI (Figure 3). For all treatments, the flag leaf had higher values than those in the 2nd leaf, and the values in 2nd leaf were higher than that in 3rd leaf. The SPAD values decreased as time. The LMF kept higher values as compared with other treatments followed by SAP. NI showed the lowest values in all season.

Figure 4 shows the relative leaf water content in flag leaf in different growing stages. The water contents were highest in jointing stage and decreasing as time. The differences among treatments were not large except for NI. NI showed lower values, while LMF and SAP had higher values. The actual quantum efficiency of PSII showed higher values in heading stage (Figure 4). Values were in jointing stage and grain-filling stage, indicating that the flag leaf of winter wheat had strong ability in photosynthetic ability in heading stage. The NI showed the lowest values in photosynthetic ability in grain-filling stage. Large differences were not found among treatments in other stages.

Yield and yield components

Yield and yield components are shown in Table 2. NI showed the lowest yields in both years (0.333 and 0.293 kgm^{-2}, respectively). Significant differences were not found among the other treatments in 2004-2005, the yields varied from 0.511 to 0.541 kgm^{-2}. On the contrary, the yields had significant differences in 2005-2006. The highest yield was obtained by LMF, WAP obtained the lowest yield. The yields of all treatments in 2005-2006 were higher than those in 2004-2005. The yields were 27, 24, 20, 19, 13 and 12% lower in 2005-2006 than those in 2004-2005 for WAP, SST, CI, WSI, LMF and NI, respectively.

NI had the lowest values in spikes number in both years (430.4 and 426.1 spikes m^{-2}, respectively).

Significant differences were not found among the other

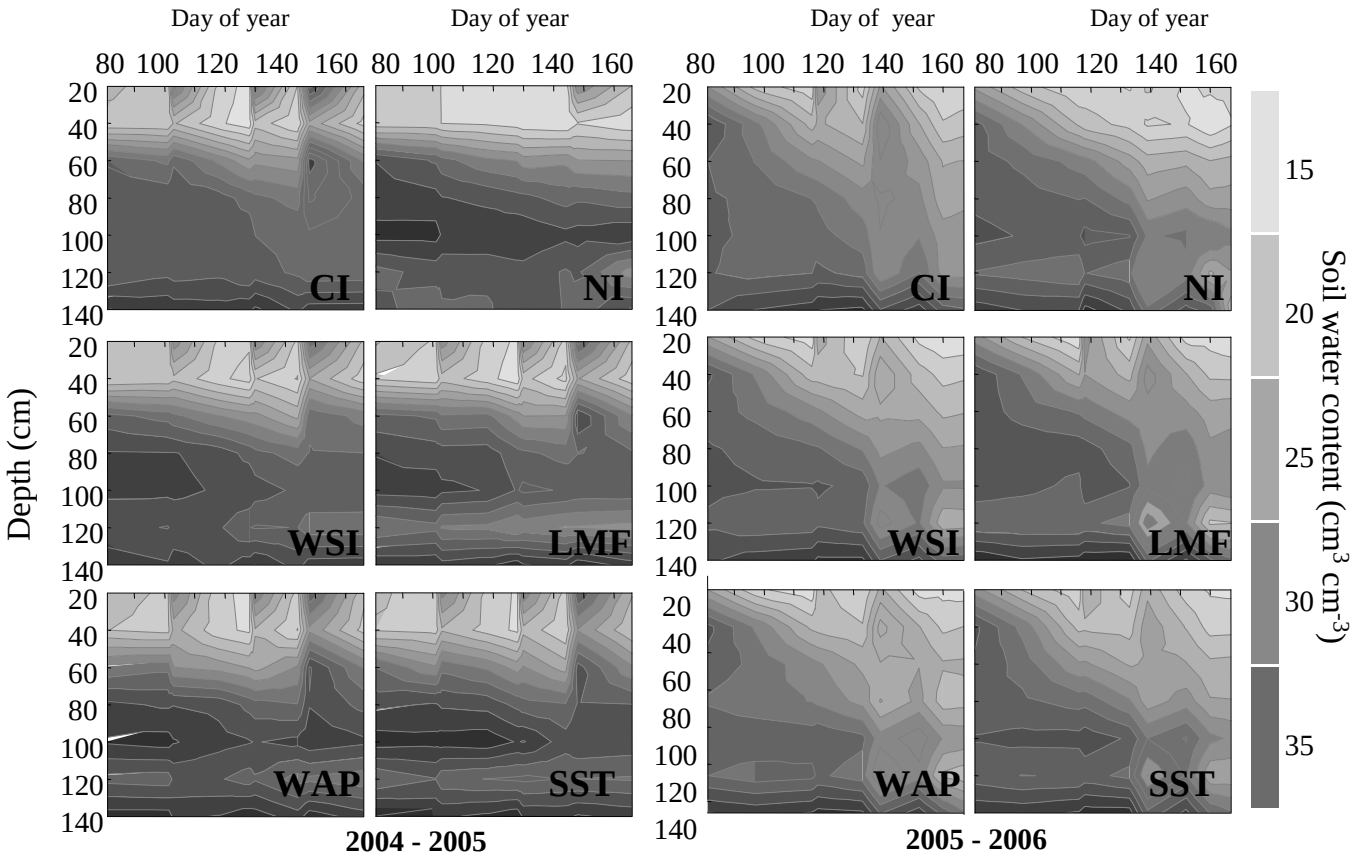

Figure 2. Changes of water content at soil profile in different treatments.

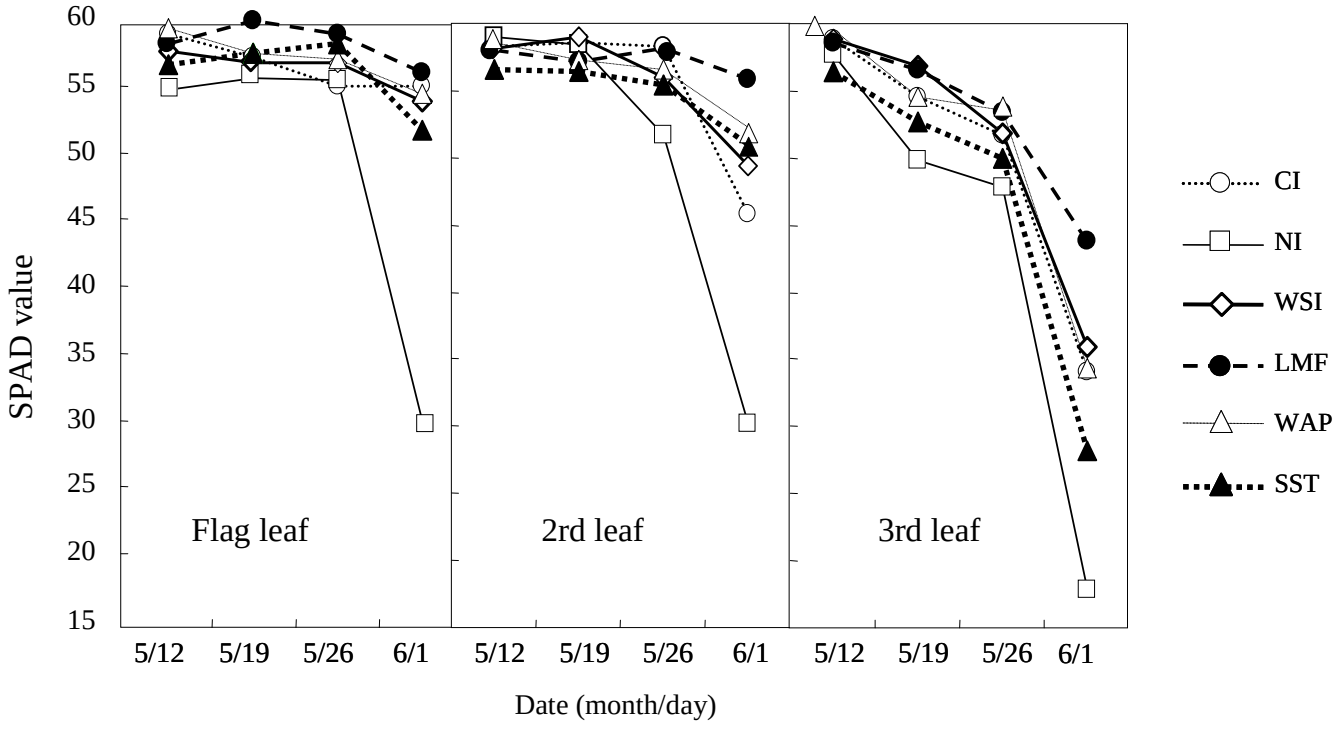

Figure 3. Changes in chlorophyll content of leafs from top in different treatments at different stages in 2004-2005.

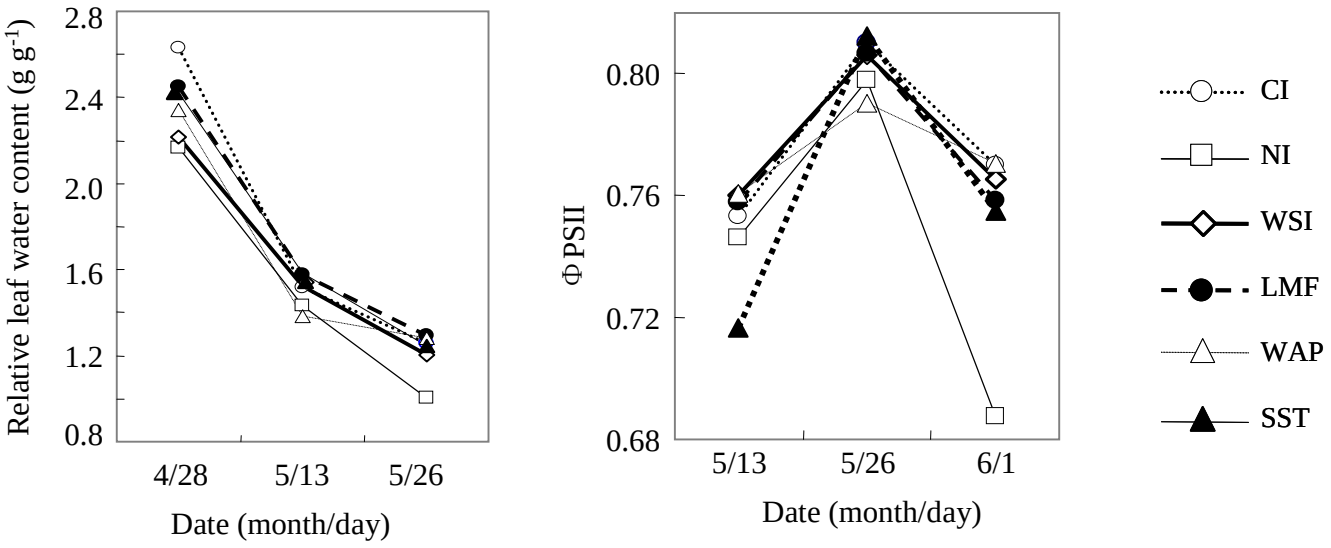

Figure 4. Changes in relative water content of flag leaf and the maximal photosynthetic quantum efficiency in different treatments at different stages in 2004-2005.

Table 2. Yield and yield components.

Treatment	Spikes number (no m^{-2})		Grain number (no $spike^{-1}$)		One hundred Kernel weight (g)		Yield (kg ha^{-1})	
	2004-2005	2005-2006	2004-2005	2005-2006	2004-2005	2005-2006	2004-2005	2005-2006
CI	543.5^{b}	517.4^{b}	34.1^{b}	28.0^{ab}	43.2^{a}	35.1^{a}	0.540^{b}	0.431^{cd}
NI	430.4^{a}	426.1^{a}	29.1^{a}	24.0^{a}	43.8^{ab}	34.5^{a}	0.333^{a}	0.293^{a}
WSI	534.8^{b}	527.5^{b}	31.0^{ab}	29.4^{b}	43.7^{ab}	36.4^{ab}	0.541^{b}	0.395^{bc}
LMF	576.8^{b}	520.3^{b}	32.9^{ab}	27.5^{ab}	46.9^{b}	41.1^{b}	0.534^{b}	0.464^{d}
WAP	526.1^{b}	488.4^{ab}	32.1^{ab}	27.2^{ab}	45.0^{ab}	39.2^{ab}	0.533^{b}	0.388^{b}
SST	552.2^{b}	543.5^{b}	31.9^{ab}	28.6^{b}	43.8^{ab}	39.1^{ab}	0.511^{b}	0.384^{b}

Different mean significant difference at 0.05 level.

five treatments in both years. The values varied from 526.1 to 576.8 spikes m^{-2} in 2004-2005 and from 488.4 to 543.5 spikes m^{-2} in 2005-2006, respectively. The values in 2005-2006 were lower than those in 2004-2005, but less than 10% for all treatments and the difference was not significant among treatments.

The same trend was found in grain numbers per $spike^{-1}$. NI had the lowest values in both years (29.1 and 24.0 grains $spike^{-1}$). The difference was not large among other 5 treatments in both years too. The values varied from 31.0 to 34.1 grains $spike^{-1}$ in 2004-2005 and from 27.2 to 29.4 grains $spike^{-1}$ in 2005-2006, respectively. The values in 2005-2006 were lower than those in 2004-2005, and the difference was significant between years.

The one-hundred kernel weight of CI was the least (43.2 g) in 2004-2005, and that of CI and NI was the least in 2005-2006 (35.1 and 34.5 g). The highest value was obtained by LMF in both years (46.9 and 40.1 g). Significant difference was not found among other 5 treatments in both years. The values in 2005-2006 were lower than those in 2004-2005. The values were 18～21% lower for CI, NI and WSI, 10～14% lower for LMF, WAP and SST in 2005-2006 as compared with those in 2004-2005, respectively.

Dry matter production and water use efficiency (WUE)

The dry matter production and allocation data was only collected in 2004-2005. CI, WSI and WAP had the most dry matter production, followed by LMF and SST, NI had the least value (Table 3). WSI had the highest value in the ratio of spike to leaf and stem, significant difference was not found among other 5 treatments. In 2004-2005, the WUE was the highest in NI, and no difference was found among other 5 treatments. In 2005-2006, NI had the highest WUE, while WAP and SST had the lowest WUE. The CI and NI were higher, WSI and LMF were equal, and WAP and SST were lower in WUE in 2005-2006

Table 3. Dry matter allocation ratio and water use efficiency.

Treatment	Total dry matter and stem ratio (gm^{-2})		Spike to leaf (kg DM mm^{-1})		WUE (kg grain mm)			
	2004-2005	2005-2006	2004-2005	2005-2006	2004-2005	2005-2006	2004-2005	2005-2006
CI	1958.5^{c}	-	1.58^{b}	-	5.49^{a}	-	15.14^{a}	17.55^{b}
NI	1167.5^{a}	-	1.61^{b}	-	7.46^{b}	-	21.28^{b}	27.30^{c}
WSI	1901.1^{c}	-	1.37^{a}	-	6.14^{ab}	-	17.44^{a}	17.27^{b}
LMF	1713.1^{b}	-	1.78^{b}	-	5.54^{a}	-	17.27^{a}	17.37^{b}
WAP	1950.8^{c}	-	1.67^{b}	-	6.26^{ab}	-	17.11^{a}	14.31^{a}
SST	1698.0^{b}	-	1.79^{b}	-	5.74^{a}	-	16.14^{a}	14.48^{a}

The different letters mean significant difference at 0.05 level. CI, Conservational irrigation; NI, non irrigation; WSI, water saving irrigation; LMF, liquid mulching film; WAP, water absorbent polymers; SST, subsoiling tillage. WUE, Water use efficiency.

as compared with those in 2004-2005, respectively.

DISCUSSION

The water consumption of winter in 2005-2006 was less than those in 2004-2005, but difference existed among treatments. It was less 30% for CI, 20% for WSI and 10% for LMF, WAP and SST, respectively. In water use efficiency, the CI and NI were higher, WSI and LMF were equal, and WAP and SST were lower in 2005-2006 as compared with those in 2004-2005, respectively indicating that only LMF could use the extra water for yield, while both WAP and SST did not help in yield producing. As all kind of mulch could help in protecting transpiration from soil surface (Chen et al., 2007; Li et al., 2008b), so more water could be used for winter wheat growth in LMF treatment instead of losing to air, which might happened in WAP and SST treatments, although, WAP could absorb more water from water, and SST method could also concentrate more water to upper soil profile. The higher soil water content in LMF treatment are also consistent with the above discussion. In our experiment, WAP did not help in yield producing both in wet condition (2004-2005) and/or in dry condition (2005-2006). SST also did not show benefit for yield producing, though water content did not show lower values as compared with those in 2004-2005. The water consump-tion is concentrated in the upper 50 cm, indicating that the soil transpiration may much stronger in dry meteorological condition for winter whea field as compared with in wet condition.

On the other hand, though water supply is sufficient in 2004-2005, our experiment showed that both LMF and WAP treatments could help in maintaining leaf chlorophyll content and leaf water content in late growth stage. It is reported that leaf chlorophyll content and leaf water content could help in maintaining photosynthetic ability in late growing periods under deficit irrigation (Maria et al., 2005; Surahmanyam et al., 2006; Zhang et al., 2006; Li et al., 2008b; Schahram et al., 2008). Furthermore, more dry matter partitioning to reproductive organs is observed in LMF and WAP treatments. It is reported that WAP could help in yield producing when water supply is optimal or water stress is light, while no benefit for yield producing when water stress is severe (Yu et al., 2006; Zhang et al., 2007; Rieger et al., 2008). Thus, LMF might be favorable for yield when grown under lower soil moisture conditions, while the application of WAP might not help in yield producing in field both in high or low soil moisture conditions. Thus, a reasonable irrigation quantity may need when applying WAP, while LMF could be used in any meteorological and/or soil water conditions. Further research of LMF when applied on crops at different soil moisture levels should be done.

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Full Length Research Paper

Heritability, variance components and genetic advance of some yield and yield related traits in Ethiopian collections of finger millet (*Eleusine coracana* (L.) Gaertn.) genotypes

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Eighty-eight (88) finger millet (*Eleusine coracana* (L.) Gaertn.) germplasm collections were tested using augmented randomized complete block design at Adet Agricultural Research Station in 2008 cropping season. The objective of this study was to find out heritability, variance components, variability and genetic advance for some yield and yield related agronomic characters. Statistically significant ($p<0.01$) difference was observed among the genotypes tested for important characters indicating the presence of variability. A considerable amount of variability among germplasms for the traits studied also indicated the usefulness of selection for these traits in the genetic material used for future improvement in finger millet. In addition, high genotypic coefficient of variation (GCV) was recorded for number tillers per plant (71.93), number of ears per plant (96.55), number of fingers per ear (85.48), finger length (94.48), biomass yield (87.67), and grain yield (78.17) and high phenotypic coefficient of variation (PCV) was similarly recorded for number tillers per plant (30.42), number of ears per plant(45.55), number of fingers per ear (24.88), finger length (26.18), biomass yield (85.56), and grain yield (29.87). High heritability coupled with high expected genetic advance as percent of mean was obtained for number of ears per plant (96.55, 90.59%), number of finger per ear (85.48, 43.81%), finger length (94.48, 50.95%), and days to heading (96.01, 14.13%), biomass yield (87.67, 154.52%), 1000 kernel weight (93.69, 37.70%), lodging susceptibility (98.92, 384.24%) and blast severity (87.60, 89.47%) indicating that the presence of more additive gene effects for potential crop improvement and so these characters could be improved through selection. This study reveals that greater yield response could be obtained through direct selection scheme in finger millet landraces.

Key words: *Eleusine coracana,* finger millet, genotypic coefficient, phenotypic coefficient, variance, heritability, genetic advance, Ethiopia.

INTRODUCTION

The presence of morpho-genetic variations in agronomic characters of a crop would be of considerable importance in determining the best method needed to improve the yield of that crop (Ojo et al., 2006). The magnitude of genetic variability present in base population of any crop species is also pivotal to crop improvement which must be exploited by plant breeders for yield improvement (Idahosa et al., 2010).

Finger millet (*Eleusine coracana* (L.) Gaertn.) is one of the most important small millet grown in eastern and southern Africa. It serves as a subsistence and food security crop that is especially important for its nutritive

and cultural value. It is an important food crop in traditional low input cereal-based farming systems in Africa, and is of particular importance in upland areas of Eastern Africa, where it commands a high market price compared with other cereals (National Research Council, 1996). In Ethiopia, traditionally it is used for making bread, 'injera' mixed with tef, porridge, local beer 'tella' and a powerful distilled sprit 'areke' and a number of other uses. Finger millet grain has good taste and is an excellent dietary source of methionine (an amino acid lacking in the diets of hundreds of millions of the poor who live on starchy foods like cassava, plantain, polished rice, and maize meal) whose level ranges around 5% of protein; is of special benefit, notably for those who depend on plant foods for their protein.

Creation of genetic variability and selection for important traits is a crutial activity that any plant breeder should apply to achieve better yield and other desirable agronimic traits. However, to carry out effective selection, the information on available genetic variation among finger millet genotypes, the nature of component traits on which selection would be effective and the influence of environmental factors on each trait need to be known (Jaleta et al., 2011). Information on the nature and magnitude of variability and heritability in a population is one of the prerequisites for successful breeding program in selecting genotypes with desirable characters (Dudly and Moll, 1969). It is therefore, of great importance for breeders to know the heritability of the agronomical characters to imrove the yield of the crop effectively.

According to Falconer and Mackay (1996), heritability is defined as the measure of the correspondence between breeding values and phenotypic values. Thus, heritability plays a predictive role in breeding, expressing the reliability of phenotype as a guide to its breeding value. It is the breeding value which determines howmuch of the phenotype would be passed onto the next generation (Tazeen et al., 2009). There is a direct relationship between heritability and response to selection, which is reffered to as genetic advance. High genetic advance with high heritability estimates offer the most effective condition for selection (Larik et al., 2000). The utility of heritability therefore increases when it is used to calculate genetic advance, which indicates the degree of gain in a character obtained under a particular selection pressure. Thus, genetic advance is yet another important selection parameter that aids breeder in a selection program. Knowledge of the extent and pattern of variability, heritability of the trait and genetic gain present in a population of finger millet collections under diversified agro-climatic condition of Ethiopia is limited where finger millet is a major food crop and farmers commonly use their landraces. Hence, this study was done with the objective to assess the variability, heritability and genetic advance of grain yield and some of its related components to select a more desired trait that may contribute for the improvement of finger millet.

MATERIALS AND METHODS

Experimental site and design

The experiment was conducted on 88 collections of finger millet germplasms including the local and standard checks obtained from the Ethiopian Institute of Biodiversity and Conservation in 2008 at Adet Agricultural Research Center, Northwest Ethiopia. Adet is located at a longitude from 37° 28' 38'' to 37° 29' 50'' E and latitude from 11° 16' 19'' to 11° 17' 28'' N in northern highlands of Ethiopia with an average altitude of 2240 masl with average annual rainfall of 1177 mm during the study and the annual minimum and maximum temperatures varied from 24.3 to 26.6°C and 8.49°C to 11.0°C, respectively. The experimental design used was augmented randomized complete block design of four blocks.

Each accession was assigned to plots of 5 m long double and sown 0.75 m apart and drilled in the row length. The seed and fertilizer rates used were 10 and 46/41 kg ha^{-1} N/P_2O_5, respectively. Hand-weeding and normal management practices were followed. Data collection was done on plant and plot basis as; plant height (cm), number of effective tillers per plant, number of ears per plant, number of fingers per ear, finger length (cm) were recorded on plant basis while days to flowering, days to maturity, biomass yield per plot (g), grain yield per plot (g), harvest index per plot (%), thousand kernel weight (g), lodging susceptibility and blast severity were recorded on plot basis. Five plants were selected at random for data record on plant basis.

Statistical data analysis

Estimation of magnitude of variation

The mean value of the recorded data was subjected to analysis of variance (ANOVA) using the statistical analysis procedures of Sharma (1998). The phenotypic and genotypic variances were also estimated according to the method suggested by Burton and De Vane (1953) using the formula:

$$\sigma^2{}_g = \frac{(MS_g - MS_e)}{r}$$

$$\sigma^2{}_P = \sigma^2{}_g + \sigma^2{}_e$$

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Abbreviations: PH, Plant height (cm); **TPP,** number of tillers per plant; **EPP,** number of ears per plant; **FL,** finger length (cm); **DH,** days to 50% heading; **DM,** days to 50% maturity; **BMY,** biomass yield per hectare; **GYPH,** grain yield per hectare(kg); **HI,** harvest index (%); **TKW,** thousand kernel weight(g); **LO,** lodging (%); **HBL,** head blast severity; **DF,** degree of freedom; **SEM,** standard error of mean; **CV,** coefficient of variation (%); **s^2p,** phenotypic variance; **s^2g,** genotypic variance; **PCV,** phenotypic coefficient of variation; **GCV,** genotypic coefficient of variation; **h^2,** broad sense heritability; **GA,** expected genetic advance; **GAM,** genetic advance as percent of the mean.

$$\sigma^2_e = MS_e$$

Where, σ^2_g =genotypic variance, σ^2_p = phenotypic variance, σ^2_e =environmental variance, MS_g = Mean square due to genotypes/accessions, MS_e = Error mean square, and r = number of replications

The coefficient of variations at phenotypic and genotypic level variation was estimated using the formula adopted by Johnson et al. (1955) as:

$$PCV = [\sigma_p / \bar{x}]x100$$
$$GCV = [\sigma_g / \bar{x}]x100$$
$$ECV = [\sigma_e / \bar{x}]x100$$

Where, σ_p = phenotypic standard deviation ($\sigma_g + \sigma_e$), σ_g = genotypic standard deviation, σ_e = Environmental standard deviation, and $\bar{x}$ = Grand mean for the characteristic x; PCV, GCV, and ECV = phenotypic, genotypic and environmental coefficient of variation, respectively.

Estimate of heritability and expected genetic advance

Heritability (h^2) in broad sense for all characters was computed using the formula adopted by Allard (1960).

$$h^2 = [\sigma^2_g / \sigma^2_p]x100$$
$$\sigma^2_p = \sigma^2_g + \sigma^2_e$$

Where, σ^2_g = genotypic variance, σ^2_p = Phenotypic variance, σ^2_e = error variance. Genetic advance as part of the mean (GA) for each character was computed using the formula by Allard (1960).

GA = (k) (σ_P) (h^2), and GAM (as % of the mean) = $\left[\frac{(GA)}{\bar{X}}\right]X100$

Where, k = selection differential (at 5% selection intensity), σ_P = phenotypic standard deviation, h^2 = heritability and $\bar{X}$ = grand mean.

RESULTS AND DISCUSSION

Mean and range

For each of the traits evaluated, the descriptive statistics including the extreme genotype mean values and the means together with their standard errors obtained on the basis of average data are summarized in Table 1. In general, finger millet genotypes showed wide range of variability for most of the characters and all the traits exhibited broad spectrum of ranges between the maximum and minimum genotype mean values. For instance, days to heading ranged from 91 to 128 with a mean of 115 days to maturity ranging from 157 to 182 with a mean of 169. Similarly, number of tillers per plant and ears per plant ranged from 4 to 14 and 5 to 32, respectively while plant height varied from 63.8 cm to111.5 cm with a mean height of 86.9 cm. Number of fingers per ear ranged from 5 to 14 with a mean of seven fingers per ear finger length of the test varieties varied from 3.9 to 12.3 cm with mean of 6.7cm.

Grain yielding ability ranged from 860.6 to 3781.5 kg ha^{-1} with a mean of 1931.8 kg ha^{-1} and that of thousand kernel weights ranged from 1.8 to 4.6 g with a mean weight of 3.3 g. The maximum yield obtained was 3781.5 kg ha^{-1} followed by 3423.6 kg ha^{-1}. Thus, it is possible to succeed in improving grain yield by direct selection.

Biomass yield ranged from 4266.7 to 15066.7 kg ha^{-1} with a mean of yield of 7637.3 kg ha^{-1} whereas harvest index varied from 15.3 to 40.8% with a mean of 25.3%. The range of variation was wide for the number of tillers and ears per plant, fingers per ear and finger length.

The result of analysis of variance on 13 quantitative characters for the genotypes is presented in Table 2. Mean square of all the characters studied, showed significant difference (P< 0.05) among the tested genotypes except for plant height and biomass yield indicating the presence of variability which can be exploited through selection.

Estimates of variance components

Grain yield, biomass yield, plant height, number of ears per plant, days to heading and maturity has exhibited high genotypic (s^2g) and phenotypic (s^2p) variances. Grain yield, biomass yield, number of ears per plant, number of tillers per plant, finger length, number of fin-gers per ear and blast severity exhibited high genotypic (GCV) and phenotypic (PCV) coefficient of variances (Table 3).

Phenotypic coefficient of variability (PCV) values ranged from 1.68% for harvest index to 188.55% for lodging, whereas the genotypic coefficient of variability (GCV) ranged from 1.18% for harvest index to 187.54% for lodging. In addition, PCV value was generally higher than their corresponding GCV values for all the characters considered (Table 3). According to Deshmukh et al. (1986), PCV and GCV values roughly more than 20% are regarded as high, whereas values less than 10% are considered to be low and values between 10 and 20% to be medium. Based on this delineation, PCV value was low for days to maturity, days to heading and harvest index; medium for plant height, and thousand kernel weight; high for number tillers per plant, ears per plant, fingers per ear, finger length, biomass yield, grain yield and blast severity.

Genotypic coefficient of variability (GCV) values were low for plant height, days to maturity, days to heading

Table 1. Ranges, means and standard errors of means (SEM) for 13 quantitative traits of 88 finger millet germplasms.

Trait	Minimum value	Maximum value	Average/mean value	SEM (±)
PH	63.8	111.5	86.89	0.95
TPP	4.0	14.0	7.24	0.21
EPP	5.0	32.0	11.53	0.52
FPE	5.0	14.0	7.01	0.16
FL	3.9	12.3	6.67	0.17
DH	91	128	114.87	0.75
DM	157	182	169.14	0.54
BMY	4266.7	15066.7	7637.33	207.39
GY	860.58	3781.48	1931.82	56.99
HI	15.31	40.82	25.49	0.45
TKW	1.80	4.60	3.26	0.06
LO	0.286	89.714	13.76	2.99
HBL	20.0	77.78	31.77	1.44

PH, Plant height (cm); TPP, number of tillers per plant; EPP, number of ears per plant; FL, finger length (cm); DH, days to 50% heading; DM, days to 50% maturity; BMY, biomass yield per hectare; GYPH, grain yield per hectare(kg); HI, harvest index (%); TKW, thousand kernel weight(g); LO, lodging (%); HBL, head blast severity; SEM, standard error of mean.

Table 2. Mean squares from analysis of variance of 13 quantitative characters of 88 finger millet germplasms.

Trait	Mean square							
	Block (DF=3)	Entries (DF=87)	Varieties (DF=83)	Checks (DF=3)	Checks vs. Varieties (DF=1)	Error (DF=9)	Total (DF=99)	(CV)
PH	52.99	94.54	95.81	152.41	184.31	43.44	88.64	7.59
TPP	3.42	4.85*	3.69*	25.75**	38.93**	1.36	4.49	16.11
EPP	34.06	27.58**	17.42**	254.73**	189.45**	0.95	25.36	8.46
FPE	0.17	3.04**	2.75**	2.83*	28.16**	0.44	2.72	9.51
FL	0.04	3.05**	2.64**	14.66**	2.14**	0.16	2.70	6.07
DH	4.52	67.38**	56.10**	298.62**	309.88**	2.69	59.59	1.43
DM	11.15	31.65**	29.38*	98.17**	20.86	6.85	28.77	1.55
BMY	3672216.0	42703311.44**	4121534.0	3983331.00	17480766.00	5266293.0	38117.18	30.05
GY	225169.00	332888.00**	283348.00*	1412120.00**	1206981.00**	72657.00	305966.0	13.95
HI	0.031	0.184	0.163	0.801**	0.018	0.097	0.171	24.49
TKW	0.13549	0.40561**	0.28597**	1.28904**	7.68528**	0.02984	0.363263	5.30
Lodging	6.770	673.140**	357.410**	6695.800**	8810.460**	7.250	592.410	6.73
HBL	15.850	248.103**	232.390**	332.260**	1300.000**	30.770	221.308	17.46

*Significant at probability level of 0.05 and **significant at probability level of 0.01. DF, Degree of freedom; CV, coefficient of variation (%).

and harvest index; medium for thousand kernel weight; high for number of tillers per plant, number of ears per plant, number of fingers per ear, finger length, biomass yield, grain yield and blast severity (Table 3). The high GCV values of these characters suggest that the possibility of improving these trait through selection.

The difference between PCV and GCV values was high for plant height, number of tillers per plant, number of fingers per ear, biomass yield, grain yield and blast severity indicating the influence of environment on these characters. However, this difference was low for number of ears per plant, finger length, days to heading, days to maturity, thousand kernel weights, harvest index and lodging suggesting minimal influence of environment on the expression of the characters, thereby having the highest estimates of heritability. Similar result was found by Yucel et al. (2006) for days to flowering, plant height and harvest index.

Estimation of heritability in broad sense and genetic advance

Estimates of heritability in broad sense ranged from 48.97% for harvest index to 98.92% for lodging (Table 3). According to Singh (2001), if heritability of a character is very high, say 80% or more, selection for such characters could be fairly easy. This is because there would be a close correspondence between the genotype and the

Table 3. Estimates of phenotypic (s^2p), genetic (s^2g) variance, phenotypic (PCV) and genotypic (GCV) coefficients of variation, heritability (H), genetic advance (GA) and GA as percentage of mean (GAM) of 13 traits of 88 finger millet germplasms.

Trait	s^2p	s^2g	PCV (%)	GCV (%)	h^2 (%)	GA	GAM
PH	94.54	51.1	11.19	8.23	54.05	10.83	12.46
TPP	4.852	3.49	30.42	25.80	71.93	3.26	45.08
EPP	27.5815	26.63	45.55	44.76	96.55	10.45	90.59
FPE	3.04149	2.6	24.88	23.00	85.48	3.07	43.81
FL	3.0482	2.88	26.18	25.44	94.48	3.4	50.95
DH	67.379	64.69	7.15	7.00	96.01	16.23	14.13
DM	31.6506	24.8	3.33	2.94	78.36	9.08	5.37
BMY	42703311.44	37437018.44	85.56	80.11	87.67	11801.51	154.52
GY	332888	260231	29.87	26.41	78.17	929.13	48.10
HI	0.18378	0.09	1.68	1.18	48.97	0.43	1.70
TKW	0.40561	0.38	19.54	18.91	93.69	1.23	37.70
LO	673.14	665.89	188.55	187.54	98.92	52.87	384.24
HBL	248.10299	217.33	49.58	46.40	87.60	89.47	89.47

s^2p, Phenotypic variance; s^2g, genotypic variance; PCV, phenotypic coefficient of variation; GCV, genotypic coefficient of variation; h^2, broad sense heritability; GA, expected genetic advance; GAM, genetic advance as percent of the mean.

phenotype due to the relative small contribution of the environment to the phenotype. Although, for characters with low heritability, say 40% or less, selection may be considerably difficult or virtually impractical due to the masking effect of environment. Considering this benchmark, heritability estimate was high (>80%) for number of ears per plant, number of finger per plant, finger length, days to heading, biomass yield, thousand kernel weight, lodging susceptibility and blast severity. It was moderate (40 to 80%) for the remaining quantitative characters.

Genetic advance under selection (GA) refers to the improvement of characters in genotypic value for the new population compared with the base population under one cycle of selection at a given selection intensity (Singh, 2001). Estimates of GA for grain yield was 929.13 kg ha^{-1} indicating that whenever we select the best, 5% high yielding genotypes as parents, mean grain yield of progenies could be improved by 929.13 kg ha^{-1}, that is, mean genotypic value of the new population for grain yield will be improved from 1931.33 to 2860.46 kg ha^{-1}. In the same way, it will be 21.98 for number of ears per plant, 19438.84 kg ha^{-1} for biomass yield, 25.92% for harvest index, 10.07 cm for finger length, and 10.08 for number of fingers per ear (Table 3).

Maximum genetic advance as percentage of mean (GAM) at 5% selection intensity was recorded for lodging susceptibility (384.24%), biomass yield (154.52%) number of ears per plant (90.59%) followed by blast severity (89.47), and finger length (50.95%). It was minimum for harvest index (1.70%) and days to maturity (5.37%).

According to Johnson et al. (1955), high heritability estimates along with the high genetic advance is usually more helpful in predicting gain under selection than heritability estimates alone. The present study reveals high heritability coupled with high expected genetic advance as percent of mean for number of ears per plant, number of finger per plant, finger length, and days to heading, biomass yield, thousand kernel weight, lodging susceptibility and blast severity; moderate heritability with relatively higher genetic advance for grain yield, number of tillers per plant, and grain yield. Therefore, these characters could be improved more easily than the other characters.

Conclusion

The PCV and GCV values were high for number of tillers per plant, number of ears per plant, number of fingers per ear, finger length, biomass yield, grain yield, lodging and blast severity suggesting the possibility of improving these traits through selection. The difference between PCV and GCV values was high for plant height, number of tillers per plant, number of fingers per ear, biomass yield, and grain yield and blast severity indicating high influence of the environment on the expression of these characters.

High heritability coupled with high expected genetic advance as percent of mean for number of ears per plant, number of finger per plant, finger length, and days to heading, biomass yield, thousand kernel weight, lodging susceptibility and blast severity; moderate heritability with relatively higher genetic advance for grain yield, number of tillers per plant, and grain yield. Therefore, these characters could be improved more easily than the other characters.

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Effect of sunlight shielding on leaf structure and amino acids concentration of light sensitive albino tea plant

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Light sensitive albino tea cultivar 'Jinguang' (*Camellia sinensis*) which grows albinism leaf in yellow colour, results to high level of amino acids but low levels of photosynthetic pigments including chlorophylls, neoxanthin, violaxanthin, phytoxanthin and β-carotene when it is exposed to high sunlight illumination in the summer season. In this case, the chloroplasts showed partially lysed, with few thylakoids. The leaf albinism was reverted when the leaf was shielded from direct illumination of strong sunlight. It is considered that the blocked development of chloroplast and photosynthetic pigments in the albinism leaf inhibited the biosynthesis of leaf proteins, resulting in an accumulation of free amino acids.

Key words: *Camellia sinensis*, leaf albinism, light intensity, photosynthetic pigments, amino acids, chloroplast.

INTRODUCTION

Amino acids are considered to be a group of quality and health benefit related components in tea (Yokogoshi et al., 1995; Liang et al., 2003; Liang et al., 2008). Albino tea cultivars are characterized by high concentration of amino acids (Du et al., 2006, 2008). There are two kinds of albino tea cultivars, that is, temperature sensitive one and light sensitive one. The temperature sensitive albino tea cultivars grow albinism shoots with high level of amino acids during early spring period when temperature is below 20°C, which can be used as good materials to processing quality green tea. However, in the summer and autumn seasons when the temperature is higher than 20°C, they grow green shoots resulting to decreased amino acids (Du et al., 2009). Normally, leaf materials from normal tea cultivars have low concentrations of amino acids and high concentration of polyphenols during summer and autumn seasons, resulting in low quality of green tea owing to their bitter taste. However, the light sensitive albino tea cultivars grow albinism shoots during the low temperature spring and the strong sunshine summer and autumn. These albinism shoots have high level of amino acids, which are suitable for processing quality green tea even in the summer and autumn seasons.

In the temperature sensitive albino tea cultivar, the expressions of genes encoding chlorophyll a/b-binding protein (lhcb), rubisco activase (RCA), D1 protein in the photosystem II core (psbA), terminal oxidase (TOX), and violaxanthin de-epoxidase (VDE) were suppressed at low temperature such as 15°C, resulting in low levels of chlorophylls and carotenoids and high level of amino acids, but the gene expression levels and pigment accumulation were reversible as the temperature increased to 20°C or above (Du et al., 2009). Specific random amplified polymorphic DNA (RAPD) markers for identifying temperature sensitive albino tea cultivars were developed (Wang et al., 2010). There has no report on the albinism mechanism of light sensitive albino tea

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Figure 1. Effect of sunlight shielding on albinism of tea leaf. A, Sunlight shielding treatment with white paper. B, Change in leaf colour after sunlight shielding.

cultivars. Effects of sunlight shielding on leaf structure and amino acids concentration of light sensitive albino tea cultivar 'Jinguang' were investigated in the present paper.

MATERIALS AND METHODS

Plant materials

The light sensitive albino tea cultivar *Camellia sinensis cv.* 'Jinguang' grown on Deshijia Tea Farm (Yuyao City, Zhejiang Province, China) was used in the experiment. A white sticky paper (2 × 4 cm) was stuck on the middle part of the second leaf from the apex bud of a shoot to make the leaf be partially shielded from sunlight in early July 2009 (Figure 1A). Ten (10) days late, the sunlight-shielded leaf portion reverted green colour and the other parts remained albinism (Figure 1B). The green and albinism parts of the leaf were exscinded separately for use.

Transmission electron microscopy (TEM)

The leaf was cut into pieces (2 × 2 mm) and fixed in glutaraldehyde solution (2.5%, v/v) overnight. The fixed samples were washed using 0.1 M phosphate buffer (pH 7.0) for three times and then re-fixed in 1% (w/v) OsO_4 for 2 h and washed as before. The samples were dehydrated in gradient ethanol (50, 70, 80, 90, 95 and 100%, v/v), for 15 min each step. The dehydrated samples were embedded in 50% (v/v) epoxy resin which was dissolved in acetone for 1 h, 67% (v/v) epoxy resin for 3 h and finally in pure epoxy resin overnight. The embedded samples were sectioned and stained with uranyl acetate and lead citrate for 15 min, and examined under JEM-1200EX transmission electron microscope (JEOL Ltd., Tokyo Japan) as method described by Du et al.(2008).

High power liquid chromatography (HPLC) of leaf pigments

Leaf pigments including chlorophylls *a* and *b*, neoxanthin, violaxanthin, phytoxanthin, β-carotene, were determined by HPLC. The leaf sample (0.2 g) was extracted in 10 mL acetone, centrifuged at 13,000 rpm at 4°C for 15 min. The supernatant (10 µL) was then injected into HPLC. HPLC was carried out on LC-2010A HPLC system (Shimadzu Corp., Kyoto, Japan) using a CLC-ODS column (6.0 × 150 mm) at 30°C and flow rate 1 ml min^{-1} as method described by Du et al. (2009). Mobile phase A was a mixture of acetonitrile/ acetic acid/ water (6/1/193, v/v/v) and mobile phase B was a mixture of acetonitrile/ methanol/ chloroform (150/40/10, v/v/v). The eluate was monitored at 450 nm using a Shimadzu SPD ultraviolet detector (Shimadzu Corp., Kyoto, Japan).

HPLC of amino acids

Leaf sample (0.5 g) was grounded in a mortar with 10 ml 70% (v/v) ethanol and centrifuged at 13 000 rpm at 4°C for 15 min. 100 µL of the supernatant was mixed with 100 µL $NaHCO_3$（0.5 M, pH 9.0）and 100 µL 1% (w/v) fluorodinitrobenzene (FDBN) in a 1.5-mL

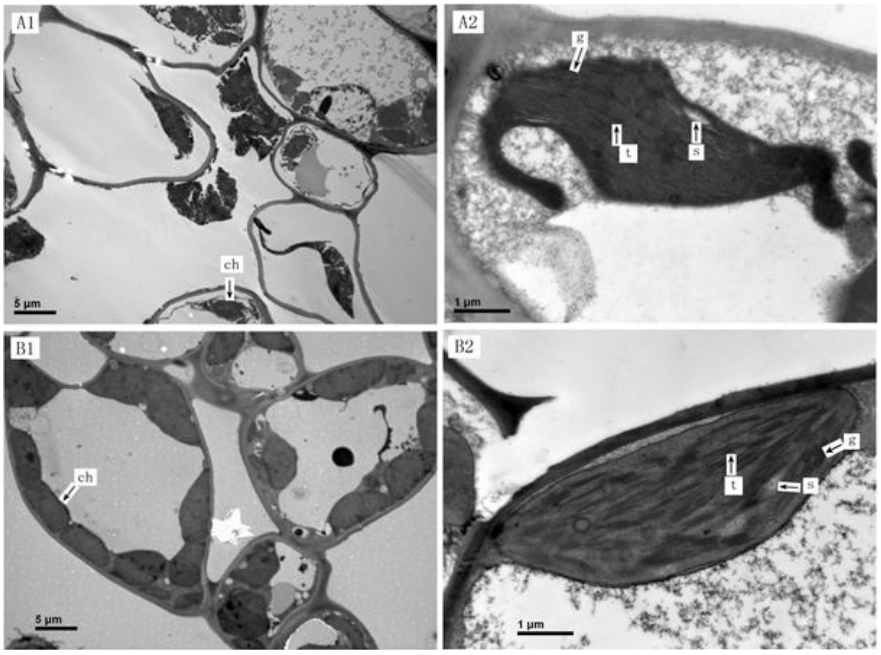

Figure 2. Ultrastructure of tea leaf. A, Leaf under natural strong sunlight. B, Sunlight shielded leaf; ch, chloroplast; g, granum; s, stroma; t, thylakoid.

centrifuge tube, heated in water bath at 60°C for 1 h. The solution was then mixed with 400 µL KH_2PO_4 (0.01 M, pH 7.0) before injected into HPLC. HPLC of amino acids was carried out on a LC-2010A HPLC system (Shimadzu Corp., Kyoto, Japan) as method described by Peris-Vicente *et al.* (2006).

RESULTS AND DISCUSSIONS

Effect of sunlight shielding on leaf albinism and ultrastructure

In the summer season, the albinism leaf of light sensitive albino tea cultivar was yellow in colour instead of green colour of normal tea plants. However, as the part of the leaf which was shielded from direct sunlight using a piece of white paper for 10 days, it resumed green colour (Figure 1). It shows that the albinism of the leaf depends on the intensity of sunlight and the albinism is convertible when the sunlight intensity is decreased.

The chloroplast is the organelle where photosynthesis occurs in photosynthetic eukaryotes. Differences in ultrastructures of natural sunlight and light shielding treatments were shown in Figure 2. There were many chloroplasts located close to the walls of the mesophyll cells in the light shielded leaf (Figure 2, B1), but few normal chloroplasts were observed in mesophyll cells of the leaf under natural strong sunlight condition (Figure 2, A1). Chloroplasts in the light shielded leaf had normal granum, thylakoid and stroma (Figure 2, B2). The chloroplasts in leaf under natural strong sunlight condition were partially lysed, in which thylakoid was few (Figure 2, A2). Chlorophylls are photosynthetic pigments which use sunlight to create sugars and thylakoid sacs are sites where chlorophyll molecules are locates. Aberrant development of chloroplasts was paralleled by impairment of chlorophyll biosynthesis (Hodgins and van Huystee, 1986), resulting in deficiency in pigments (Fambrini et al., 2004). This study shows that the disfunction of chloroplasts is consistent to the albinism of the tea leaf. The arrested development of thylakoids might block chlorophyll accumulation in leaf. It explains why the albinism phenomenon of cultivar ‘Jinguang’ occurs under high sunlight illumination in the summer. It is confirmed that photosynthetic performance of a plant can be severely inhibited following exposure to light intensities in excess of those required to saturate photosynthesis (Osmond, 1981).

Despite the essential requirement of light for the functional activity, an excessive light inhibits chloroplast electron transport, resulting in photodamages (Powles, 1984). The albinism of the light sensitive albino tea cultivar might be related to its photodamages under strong sunlight conditions in the summer.

Effect of sunlight shielding on concentration of leaf photosynthetic pigments

Leaf photosynthetic pigments including neoxanthin, violaxanthin, phytoxanthin, β-carotene and chlorophylls *a* and *b* were significantly increased when the leaf was shaded with white paper under strong sunlight condition

Table 1. Concentration of leaf photosynthetic pigments (μg g^{-1}, FW)[a].

Pigment	Natural sunlight	Shading
Neoxanthin	4.48±0.52	10.99±1.06
Violaxanthin	18.75±3.02	24.62±3.05
Phytoxanthin	54.64±0.08	110.44±8.22
β-Carotene	67.70±0.26	151.34±9.5
Chlorophyll *a*	166.05±8.21	669.16±4.31
Chlorophyll *b*	34.22±0.75	138.18±5.28
Total	200.27±8.96	807.34±9.59
Chlorophyll *a*/ Chlorophyll *b*	4.85	4.84

[a]FW: Fresh weight.

Table 2. Amino acids of albino tea cultivars during illumination treated (mg g^{-1}, FW)[a].

Amino acid	Natural sunlight	Shading
Aspartic	0.714 ± 0.052	0.252 ± 0.082
Glutamate	1.059 ± 0.022	0.618 ± 0.091
Asparagine	0.017 ± 0.004	0.015 ± 0.002
Serine	0.012 ± 0.003	0.007 ± 0.002
Histidine	0.130 ± 0.023	0.067 ± 0.008
Threonine	0.048 ± 0.005	0.033 ± 0.004
Theanine	2.117 ± 0.122	1.428 ± 0.105
Alanine	0.085 ± 0.007	0.000
Lysine	0.158 ± 0.022	0.129 ± 0.051
Valine	0.000	0.002 ± 0.001
Methionine	0.069 ± 0.004	0.029 ± 0.002
Cysteine	0.087 ± 0.006	0.000
Tryptophan	0.043 ± 0.012	0.006 ± 0.002
Phenylalanine	0.164 ± 0.021	0.070 ± 0.011
Leucine	0.119 ± 0.012	0.064 ± 0.012
Tyrosine	0.085 ± 0.005	0.014 ± 0.003
Total	4.905 ± 0.476	2.733 ± 0.257

[a:] FW, Fresh weight.

in the summer (Table 1). Compared to leaf under natural strong sunlight, level of Chlorophylls *a* and *b* in the light shielded leaf increased by four times, and those of neoxanthin, phytoxanthin and β-carotene were doubled. Violaxanthin in the light shielded leaf increased by 31%. However, there was no significant difference in the ratio of chlorophyll *a* to chlorophyll *b*, suggesting that the light shielding had a same effect on the chlorophylls *a* and *b*. The decrease in level of chlorophylls is considered to be related to the albinism of the tea leaf.

Neoxanthin, violaxanthin, phytoxanthin and β-carotene are components of carotenoids in tea leaf and they absorb light energy for use in photosynthesis and protect chlorophyll from photodamage. Carotenoids are usually considered to perform two major functions in photosynthesis. They serve as accessory light harvesting pigments, extending the range of wavelengths over which the light can drive photosynthesis, and they act to protect the chlorophyllous pigments from the harmful photodestructive reaction or photodamage which occurs in the presence of oxygen under excessive sunlight condition (Cogdell, 1978).

Photodamage to photoautotrophic organisms under unfavorable environmental conditions proceeds primarily via the increased generation of reactive oxygen species (Solovchenko and Merzlyak, 2008). It is considered that these pigments work as screening pigments when illumination is above the light saturation point, during which they were partially consumed, resulting in their low levels in the albinism leaf under natural strong sunlight condition in the summer.

Effect of sunlight shielding on concentration of amino acids

There was significant difference in amino acids concentration between natural strong sunlight and sunlight-shielded treatments (Table 2). Total concen-tration of amino acids in sunlight-shielded leaf was decreased by 44.3%, compared to that under natural strong sunlight. Theanine was the most abundant com-ponent of tea leaf amino acids and glutamate the next in the two treatments. Valine was not detected in the natural strong sunlight sample. Alanine and cysteine were not detected in sunlight-shielded sample (Table 2). This suggests that biosynthesis and accumulation of amino acids in the light sensitive albino tea plant leaf is affected by light intensity.

Photosynthesis-related plastid mRNAs and plastid tRNAs were confirmed to be down-regulated in albino barley and maize leaves (Zubko and Day, 2002). Some protein synthesis is independent on chlorophyll and carotenoid development in the plastids (Fambrini et al., 2004). A light-yellow mutant of *Plantago insulari* grown in the dark became bleached on exposure to light, yet a moderate increase in nitrogen occurred during exposure to light (Michael and Spurr, 1975). The present study shows that the development of chloroplast and photosynthetic pigments including chlorophyll, neoxanthin, violaxanthin, phytoxanthin and β-carotene was blocked in the albino leaf. Amino acids are the monomers for synthesizing proteins in plant. It is considered that the blocked development of chloroplast and photosynthetic pigments inhibited the biosynthesis of leaf proteins, resulting in accumulation of free amino acids.

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Phytase activity of fungi from oil polluted soils and their ability to degrade bonnylight crude oil

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Fungi were isolated from contaminated soil samples taken from three selected automobile workshops, screened for phytase activities and biodegradative abilities. Physicochemical and total petroleum hydrocarbon analyses were carried using standard chemical and gas chromatography procedures, respectively. There was significant increase (at P≤0.05) in the potassium, sodium, calcium, magnesium, pH and organic matter of all contaminated soil samples. The fungi isolated were *Aspergillus niger*, *Aspergillus saprophyticus, Aspergillus fumigatus, Aspergillus flavus, Trichoderma viride, Penicillium italicum, Articulospora inflata* and *Neurospora crassa*. Of all the fungal isolates, *A. flavus* had the maximum phytase activity at the 48 h of incubation while *N. crassa* produced the least phytase activity at all the hour of incubation. Phytase activity of *A. flavus* and *A. saprophyticus* were found to be most active at pH 5.0 and 50°C. *A. niger* had the highest degrading ability on crude oil and spent engine oil at all days of incubation while *N. crassa* had the least degrading ability on crude and spent engine oil. The high total petroleum hydrocarbon (TPH) concentration in contaminated soil may be as a result of consistent exposure of the soil to spent engine oil which could make the soil conditions unsatisfactory for microbial growth.

Key words: Fungi, biodegradation, bonny light crude oil, phytase.

INTRODUCTION

Crude oil accounts for approximately 35% of total global energy usage and consists of several hydrocarbons (Metman et al., 2010). Crude oil spills from pipelines and refineries leads to oil pollution which causes damage to the environment (Ogbe et al., 2006). Petroleum hydrocarbon compounds bind to different soil components and these are difficult to remove or degrade (Erdogan and Karaca, 2011). Oil pollution is a major environmental concern in many countries (Nikolopoulou et al., 2013) and this has led to a concerted effort in studying the feasibility of using oil degrading fungi and bacteria for biodegradation (Akoachere et al., 2008). Many microorganisms have the ability to utilize hydrocarbon as the sole source of carbon and energy. It is known that greater degradation of oil pollutants is carried out *in situ* by a consortium of microorganisms and more than 200 species of bacteria, fungi and even algae can biodegrade hydrocarbons (Onifade and Abubakar, 2007). The advantages associated with fungal bioremediation lay primarily in the versatility of the technology and its cost efficiency compared to other remediation technologies (Chaudhry et al., 2012).

Microbes associated with several soil samples are rich sources of new enzymes (Akpan, 2004). Phytic acids (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) are a group of organic phosphorus compounds found

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Abbreviations: MSM, Minimal salts medium; **GC,** gas chromatographic; **TPH,** total petroleum hydrocarbon.

widely in nature (Chang et al., 2004). Phytases are commonly found in nature and can be derived from a number of sources including plants, animals and microorganisms (Krovuo et al., 2002; Singh et al., 2013). There has been a great deal of interest on the study of microbial phytase production and the optimization of media and conditions for maximum production of the enzyme with the aim to increase yields to make it economical as a commercial product. There are many applications of phytic acid, including industrial use as a corrosion inhibitor on metals, a rust remover and an additive to lubricating greases, use as a food additive, and medical applications, including use in the prevention of dental caries, use as an imaging agent for organ scintography and an X-ray enhancement contrasting agent (Chu et al., 2001). It is being used as a hypocholestromic agent, used to reduce gastric secretion for treatment of gastritis, gastroduodenitis, gastric duodenal ulcers and diarrhea, and used as an antidote for toxic metal absorption. It is therapeutically used in the prevention and dilution of calcium deposits associated with various diseases and for reducing calcium concentration in urine (thus checking the formation of renal calculi), It is also used as a preventive agent against severe poisoning with pressurized oxygen and preventing thirst during exercise, as a taste-improving agent in orally administered antibiotics, and in the treatment of multiple sclerosis (Chu et al., 2001). The objectives of this study were therefore, to isolate and identity fungal flora of oil contaminated soils and determine if there would be any correlation between phytase production and degradative abilities of the indigenous fungal isolates.

MATERIALS AND METHODS

Collection of sample

The contaminated and non-contaminated soil samples were collected from three different mechanic workshops at three different spots using a soil auger. Soil samples for physico-chemical analysis were collected in polyethylene bags while those for microbiological analysis were collected in sterile screw-capped bottles. Analysis commenced immediately upon arrival in the laboratory. Unused samples were refrigerated at 4°C.

Physicochemical analysis of the soil samples

Physicochemical properties of the soil samples such as pH, total nitrogen, organic carbon, organic matter, calcium, magnesium, potassium, sodium, phosphorus and moisture content were determined according to Ibitoye (2006).

Isolation, enumeration and identification of fungi from uncontaminated and contaminated soil samples

1 g of soil sample was taken from the sterile screw-capped bottles into 10 ml sterilized water in a test tube to form stock solution. This was repeated until the 8th dilution; 1 ml of the diluents from the two to four fold dilutions were pour plated on sterilized potato dextrose agar for fungi, allowed to solidify and incubated at 28±2°C for 72 h. Colonies that developed were counted and recorded as spore forming unit per gram of soil (sfu/g) for fungi. The isolates were subcultured repeatedly to obtain pure isolates. Isolated fungi were characterised by macroscopic (physical appearance on agar plates) and microscopic techniques (under light microscope) including colour of aerial and substrate mycelia comparing them with those of known taxa (Domsch and Gams, 1970).

Preparation of inoculating medium for the growth of culture

Cornstarch medium consisted: Cornstarch (Hubinger), 80 g; glucose, 30 g; $MgSO_4$. $7H_20$, 0.5 g; KCl, 0.5 g; $FeSO_4$, 0.1 g; $NaNO_3$, 8.6 g; K_2HPO_4, 0.2 g; pH 5.0. The solution was mixed to effect complete dissolution following the addition of sodium phytase into each flask. The conical flask containing each medium was sterilized by autoclaving at 121°C for 15 min. Inoculum for the moulds was prepared by transferring 2 x 10^7 spores per ml from stock slants to 50 ml of cornstarch medium in 250-ml Erlenmeyer flasks which were incubated for 3 days at 28°C on an orbital shaker (270 rev/min).

Preparation of crude extract

A set of twelve (12) 100 ml conical flasks was labeled A to L to cover the 72 h incubation period. Each flask contained 30 ml optimized basal medium. All the flasks with their contents were sterilized in an autoclave at 121°C for 15 min, the solution was allowed to cool and inoculum for the moulds was prepared by transferring 2 x 10^7 spores per ml from stock slants was introduced, respectively, into each of the incubating flasks and then incubated at 37°C in shaking water bath for the period of 72 h. Five mililitre (5 ml) of the enzyme solution was withdrawn from each of the cultured medium after 72 h of the incubation period. The enzyme solution obtained daily was centrifuged at 6,000 rpm to get clear supernatant which will be used for phytase assay. The supernatant obtained was used for the analysis of phytase activity and the physicochemical properties of phytase.

Determination of phytase production through assay of phytase activity

Phytase activity was determined by measuring the initial rate of phosphorous, as indicated by an increase in absorbance at 415 nm. The sample test tube contained 1 ml of the enzyme solution, 2 ml of substrate solution incubated at 37°C for 65 min using a regulated Gallenhamp water bath and the reaction was stopped with 2 ml of colour stop mix to precipitate the enzymes. Blank sample contained only 1 ml of sodium acetate buffer, 2 ml of substrate and 2 ml of colour stop mix. Colour stop mix consist mixture of ammonium molybdate stock solution, ammonium vanadate stock solution, nitric acid ($HN0_3$) and distilled H_20. One enzyme unit (IU) was defined as 1 µmol phosphate liberated per minute.

Effect of pH on the activity of phytase

Two fungi, *Aspergillus saprophyticus* and *Aspergillus flavus* were selected for this assay. The optimal pH was determined by measuring the activity pH range 4.0-8.0, using 0.1 M acetate (pH 4.0-5.0), 0.1 M phosphate (pH 6.0-7.0) and 0.1 M Tris (pH8.0) buffers. Maximum activity was taken as the optimum pH for phytase for activity expressed in comparison with maximum activity (Gulati et al., 2007).

Effect of temperature on activity of phytase

The assay mixture was incubated at different temperature from 30-60°C to determine the effect of temperature on enzyme activity. At

65 min incubation period, 2 ml colour stop mix was added and the enzyme activity was measured according to the standard assay method at 5°C internal for each of the different temperature.

Determination of rates of utilization of crude oil and used engine oil by fungal isolates

Minimal salts medium (MSM) of Zajic and Supplison (1972) containing; 1% refined petroleum product (crude oil and used engine oil) as the only source of carbon, 0.27 g K_2HPO_4, 0.6 g NH_4Cl, 0.03 g $MgSO_4.7H_2O$, 0.015 g NaCl, 0.0015 g $NaSO_4.7H_2O$ was used. Crude oil and used engine oil were tested directly for the ability of fungal and bacterial isolates to degrade them using the method earlier described by Okpokwasili and Okorie (1988), as their sole sources of carbon and energy by the determination of growth turbidity. This was carried out by dispensing 100 ml of MSM into conical flask (Zajic and Supplison, 1972). Following sterilization by autoclaving and cooling, 0.1 ml of the isolates from 10^{-4} to 10^{-6} dilutions were seeded in 1,000 ml of minimal salt medium, pH 7.4, medium, followed by 0.1 ml filter-sterilized (0.45 µm pore size filter, Millipore) crude oil and used engine oil. The cultures were then incubated at room temperature for seven days. For each isolate, a control was set up in which no organism was seeded. At the end of the incubation, the optical density (OD) of each culture was measured at 650 nm (Eja et al., 2003) using spectronic 20 Genseys spectrophotometer. In this case, the OD was an index of growth reflecting the potential for the biodegradation of the petroleum products by the respective fungal species.

Characterization of crude oil and used engine oil

The crude oil sample (Bonny light), used engine, contaminated and non-contaminated soil sample were subjected to total hydrocarbon analysis using gas chromatographic (GC) method to determine total petroleum hydrocarbon (TPH) content of the crude oil and engine oil sample according to the method of Adesodun and Mbagwu (2008).

Statistical analysis

Data obtained were subjected to a single factor analysis of variance (ANOVA) while the significant means were separated with the Duncan's multiple range test (DMRT) at 5% confidence level (P = 0.05) using SPSS (16).

RESULT

Physicochemical properties of soil samples

The physicochemical properties of contaminated and uncontaminated soil sample immediately after collection are as shown in Table 1. The values of pH in contaminated soils ranged from 6.61 to 7.58 while pH of uncontaminated soils ranged from 6.40 to 6.64. The organic carbon content and organic matter ranged from 0.04 to 3.89% in contaminated soils and 0.03 to 0.20% in uncontaminated soils. The calcium concentration in contaminated soil ranged from 0.92 to 4.13 mg/kg while those in uncontaminated soils ranged from 1.70 to 2.11 mg/kg. Magnesium ion concentrations were higher in contaminated soils than in uncontaminated soils. It ranged from 0.70 to 3.22 mg/kg while those in uncontaminated soils ranged from 0.91 to 1.82 mg/kg. Sodium ion concentration in contaminated soils ranged from 0.05 to 0.42 mg/kg and it ranged from 0.10 to 0.15 mg/kg in uncontaminated soils. Potassium ion concentration in contaminated soils ranged from 0.09 to 0.41 mg/kg while those in uncontaminated soils ranged from 0.09 to 0.11 mg/100g. The phosphorus content in contaminated and uncontaminated soils ranged from 6.18 to 33.33 mg/kg and 1.99 to 3.38 mg/kg, respectively. Nitrogen level in contaminated soils ranged from 0.05 to 0.38% while in uncontaminated soils it ranged from 0.05 to 0.09%.

The results obtained from physicochemical analysis revealed that the contaminated soil contained high appreciable essential nutrients phosphorus in all the contaminated soils compared to non-contaminated soil. There was significant difference in the potassium, nitrogen; sodium, calcium, magnesium, pH, organic carbon and matter of all contaminated soil samples while there were no significant difference in that of all the non-contaminated soil samples.

Total plate count of fungi in uncontaminated and contaminated soils

The total plate count of fungi in contaminated soils ranged from 1.0×10^5 to 2.0×10^5 sfu/g, while in uncontaminated soils, it ranged from 2.0×10^5 to 3.0×10^5 sfu/g. Eight fungal isolates; *Aspergillus niger, A. saprophyticus, A. flavus, Aspergillus fumigatus*, *Trichoderma viride, Penicillium italicum, Articulospora inflata* and *Neurospora crassa* were obtained from both contaminated and non-contaminated soil samples.

Growth profile of isolated fungi

In Figure 1, there was an exponential phase at 38 to 45 h of incubation, *A. fumigatus* and *A. saprophyticus* had 18 to 45 h of incubation, *A. niger* had 40 to 60 h for incubition and *T. viride* had 22 to 50 h of incubation.

Production of phytase

Result of phytase activity from isolated fungi is presented in Figure 2. From this study, it was observed that of all the fungal isolates, *A. fumigatus* had the maximum phytase activity at 48 h of incubation while *N. crassa* produced the least phytase activity at all the hour of incubation. Phytase activity of *A. flavus* and *A. saprophyticus* were found to be most active at pH 5.0 (Figure 3). Also, at 50°C, phytase activity of *A. flavus* and *A. saprophyticus* were found to be optimum (Figure 4).

Utilization of bonny light crude oil and used engine oil by fungal and bacteria isolates

The degradative abilities of fungal isolates on crude and used engine oil are as shown in Figures 5, 6, 7 and 8.

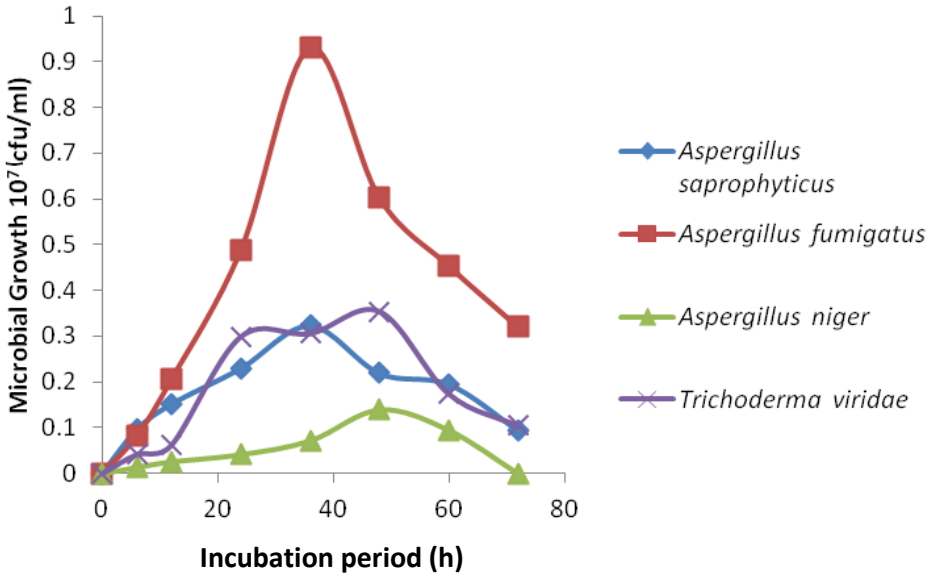

Figure 1. Growth profile of selected fungal isolates in optimized nutrient medium.

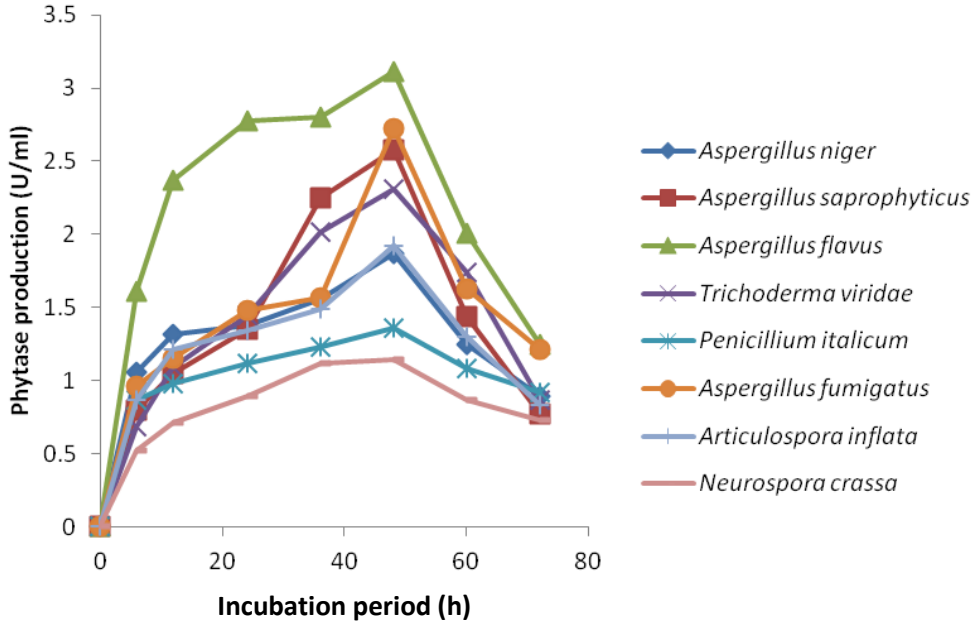

Figure 2. Phytase activity of fungal isolates.

Degradative abilities of crude oil were observed to be in the following order: *A. niger* > *A. fumigatus* > *T., viride* > *A. flavus* > *A. saprophyticus* > *A. inflata* > *P. italicum* > *N. crassa.* On the other hand, the abilities of fungi to degrade used engine oil were observed from the study to be in the following order: *A. niger* > *A. fumigatus* > *A. saprophyticus* > *T. viride* > *A. flavus* > *P. italicum* > *A. inflata* > *N. crassa. A. niger* was observed to be the best fungus for rapid degradation of crude oil and used engine oil while *N. crassa* was the least degrader of crude oil and used engine oil at all time of incubation.

Total petroleum hydrocarbon content of the crude oil and soil sample

The total petroleum hydrocarbon content showed that soil sample A had the highest with a total of 856,328 Mg/kg,

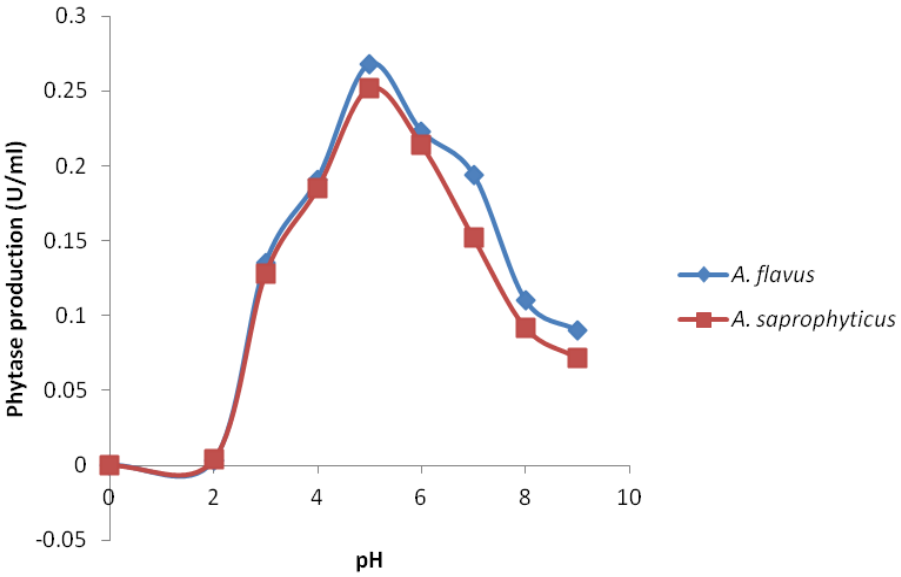

Figure 3. Effect of pH on the activity of phytase from *A. flavus* and *A. saprophyticus*.

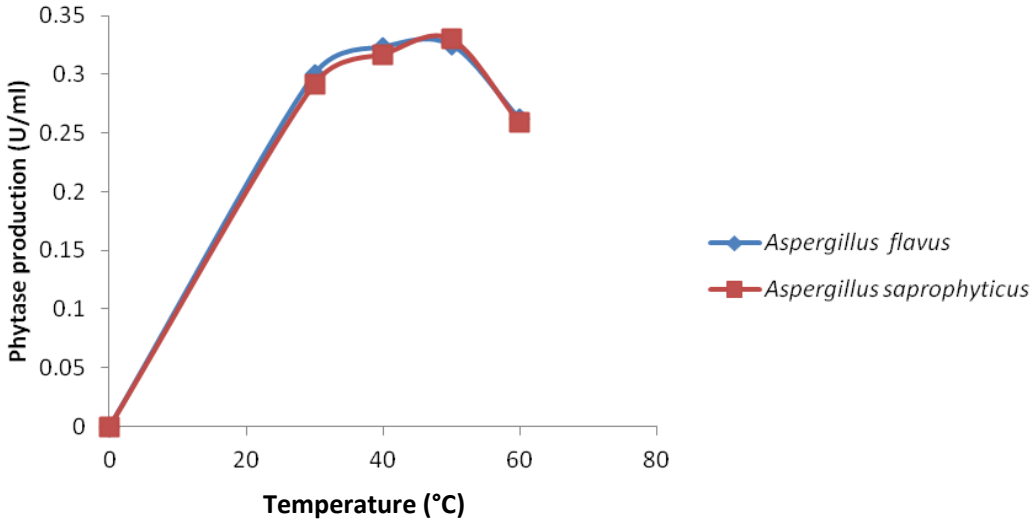

Figure 4. Effect of temperature on the activity of phytase from *A. flavus* and *A. saprophyticus.*

sample B had 642,302 Mg/kg sample C had the least with 545, 168 Mg/kg, that of the uncontaminated sample was 4.90 Mg/kg, while that of the bonny light crude oil and used engine oil was 98,346.102 Mg/l and 35,726.80 Mg/l.

DISCUSSION

The relative high pH in contaminated soil as compared to the non-contaminated soil could be as a result of the production of some organic acids in the course of the biodegradation process (Dennis, 2009). The slow biodegradation of organic compounds is often associated with low concentration of one or more inorganic nutrients needed for microbial growth in natural environments (Lewis et al., 1986; Swindoll et al., 1988; Coveney and Wetzel, 1992). The addition of nitrogen and phosphorus may increase the biodegradation of a compound (Pritchard and Costa, 1991). The results obtained revealed that the contaminated soil contained appreciable essential nutrients like nitrogen, phosphorus, potassium, magnesium, calcium and sodium. The calcium and magnesium ion concentrations in contaminated soils were higher than those in uncontaminated soils. This is believed to be the action of phytase on the phosphorus complexes, known as phytate.

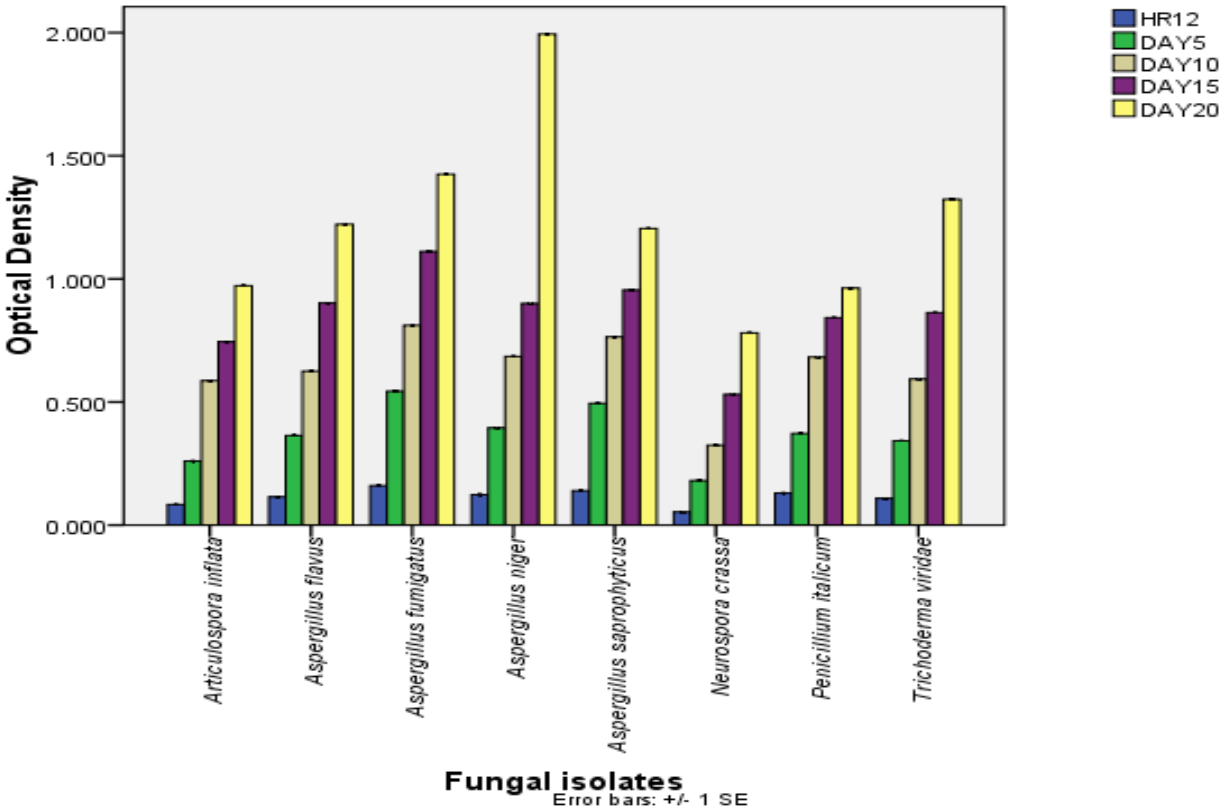

Figure 5. Optical density of fungal isolates from contaminated soil at different hours of biodegradation of bonny light crude oil.

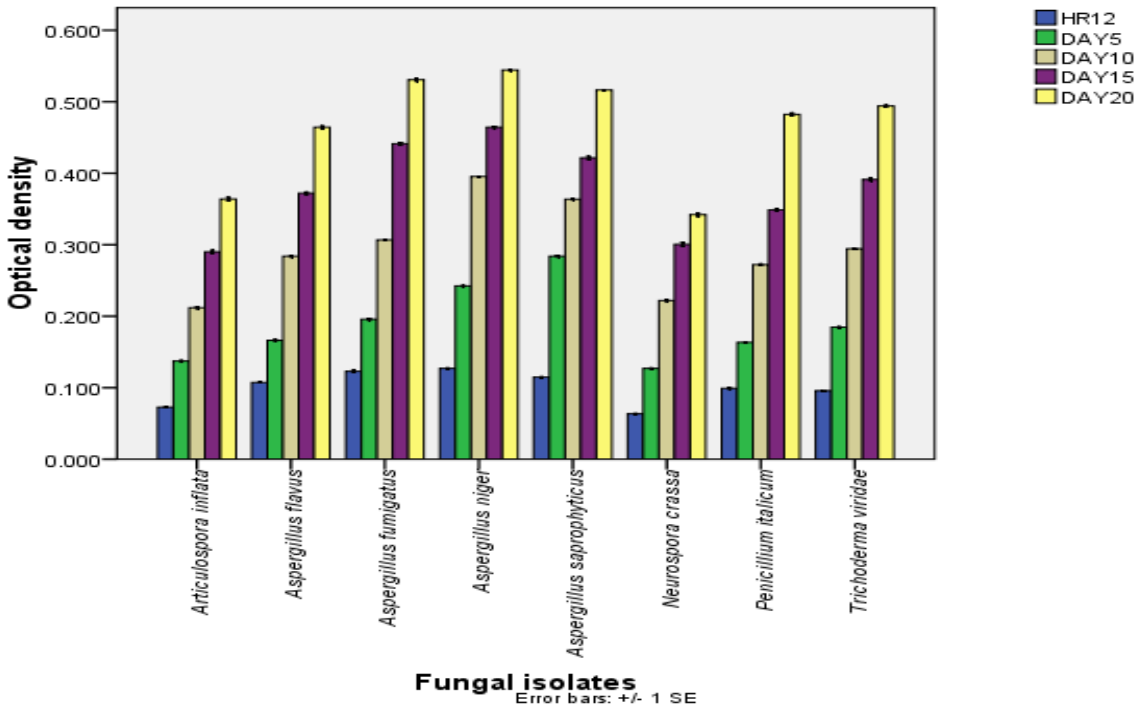

Figure 6. Optical density of fungal isolates from contaminated soil at different hours of biodegradation of used engine oil.

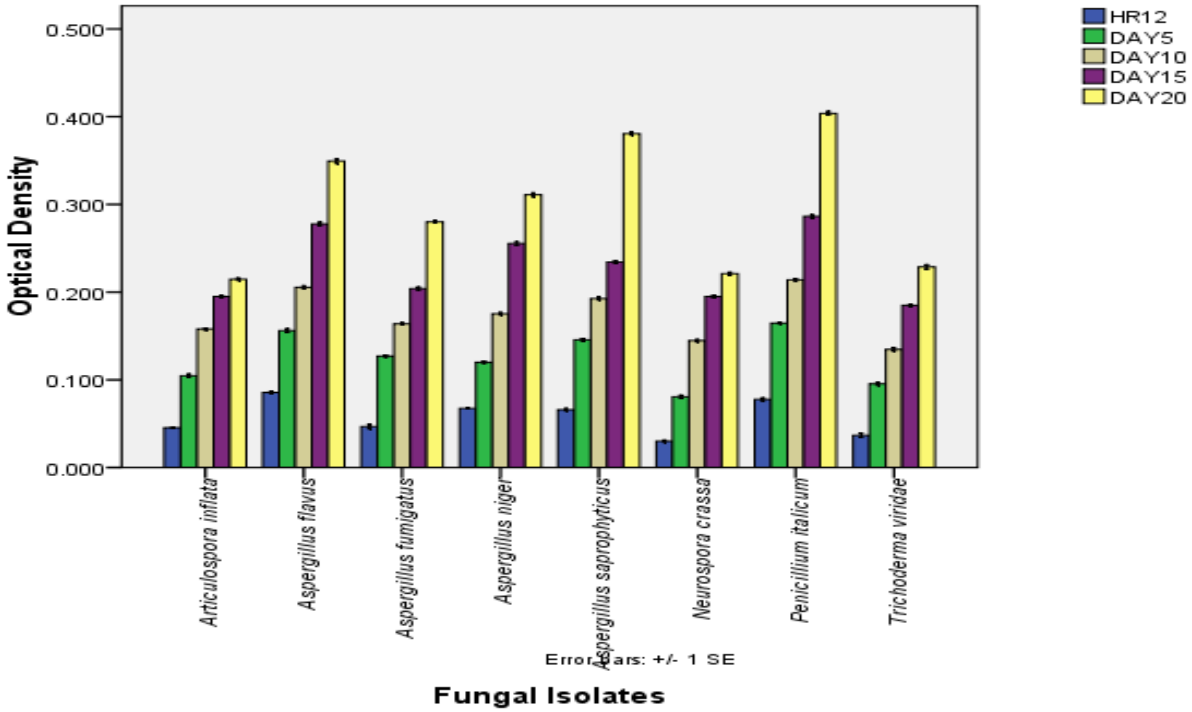

Figure 7. Optical density of fungal isolates from non-contaminated (control) soil at different hours of biodegradation of bonny light crude oil.

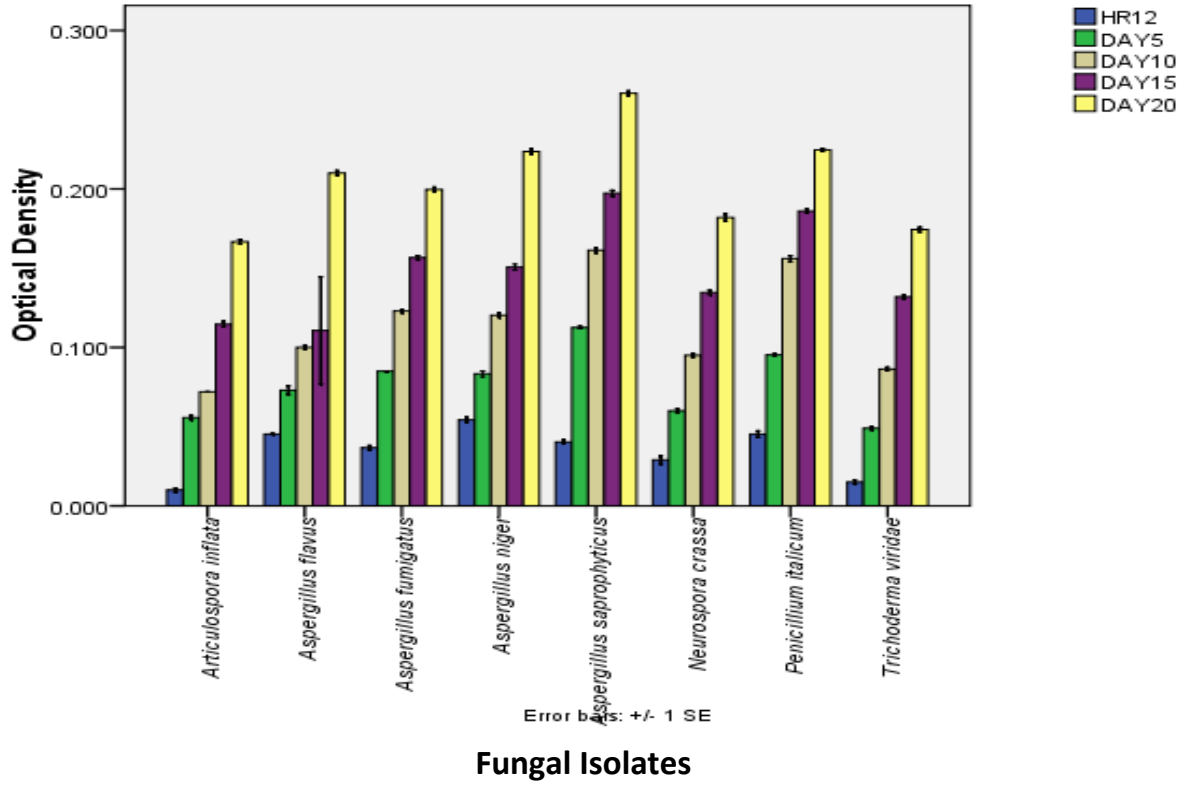

Figure 8. Optical density of fungal isolates from non-contaminated (control) soil at different hours of biodegradation of used engine oil.

This might occur as a result of phytase hydrolysing the phytate that has being chelated to release phosphate as a form of nutrient that will enhance biodegradation process. The significant differences between the concentration of potassium ion in contaminated and uncontaminated soils, indicate that the potassium ion in the contaminated soil is as a result of phytase released by the organism causing the phytate to be hydrolyzed. It was observed from this study that phosphorus concentrations in contaminated soils were higher than those in uncontaminated soils and this might occur as a result of phytase released by the organism causing the phytase to be hydrolyzed.

The total plate counts of fungi were higher in uncontaminated soils than contaminated soils. This is likely to be due to the environmental stress and toxicity caused by the hydrocarbons to the fungi. This finding agreed with the report of Atlas and Bartha (1992) that crude oil products contain hydrocarbon that are toxic to microorganisms. The result shows an obvious influence of waste engine oil and crude oil discharge on the microbiological and physiochemical properties of soil. The significant difference in the total plate count of fungi in contaminated and uncontaminated soil samples may be due to the fact that the fungi thriving in contaminated soils were able to synthesize enzymes capable of digesting the hydrocarbons in the crude oil and used engine oil (Ijah and Abioye, 2003).

The growth profile of the isolated microorganisms showed the growth curve exhibiting the lag, exponential, stationary and decline phase which agrees with the report by Prosser (1983) that the morphology and growth characteristic of the isolated microorganism vary continuously throughout colony development. The curve showed an exponential phase from 12 to 48 h. All the fungal isolated from this study were able to produce phytase in varying degrees. Kumat and Bhat (2011) found out that out of 161 fungal isolated forty soil samples, only 33 were phytase producers. Jared et al. (2010) also noted that *A. fumigatus* was among the fungal isolates that produced phytase. The maximum phytase activity was more prominent at the 48 h of incubation. It was observed that of all the fungal isolates, *A. flavus* had the maximum phytase activity at the 48 h of incubation while *N. crassa* produced the least phytase activity at all the hour of incubation.

The pH versus phytase activity profiles of the selected fungi displayed substantial production of phytase at two distinct pH optimum; the highest activity was recorded at pH 6.0 and a second activity peak occur at pH 3.0 which is in accordance with the work of Gibson (1984). Maximum activity for phytase production from the selected fungi was attained at 50°C. According to Mullaney and Ullah (2003), the phytase molecule has a limited thermal stability and studies have demonstrated that losses in activity begin to occur at around 60°C. *A. flavus, A. fumigatus, A. saprophyticus, A. niger, T. viride, N. crassa, A. inflata* and *P. italicum* isolated in this study showed evidence of high ability to degrade crude oil as compared to used engine oil and this can be attributed to the presence of saturated alkane with intermediate chain (C10 – C24) length (Atlas and Bartha, 1996). However, *N. crassa, A. inflata* and *P. italicum* showed the lowest degrading ability on crude oil and used engine oil. Fungi show tremendous diversity and adaptability in utilization of different organic molecules as a carbon source, however, their abilities to degrade a specific hydrocarbon as a source of energy and/or biomass may differ (Hadiba and Tachinaba, 2009). The ability shown by the isolates in contaminated soil is believed to have been enhanced by the sufficient availability of phosphorus which is made available by the hydrolyzing effect of phytase on the complex salts of phosphorus that are in insoluble in the soil, and phosphorus is known as one of the limiting nutrient in biodegradation process.

The high TPH concentration in contaminated soil is as a result of consistent exposure of the soil to used engine oil, this high concentrations of the TPH make soil conditions unsatisfactory for microbial growth (Dejong, 1980). The TPH level in the uncontaminated soil (control) was as low as 4.9 mg/kg indicating that hydrocarbons could be present in uncontaminated soils and sediments, as earlier reported by Geiger and Blumer (1974). This research provides information that would lead to selection of fungal species and physicochemical condition that could be employed for bioremediation of soils polluted with crude and spent engine oil. It is therefore concluded that oil-degrading fungi are abundant in soils collected from mechanic workshop in Akure.

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African Journal of Biotechnology

Full Length Research Paper

Chemical and organoleptic evaluation of fermented maize *(Zea mays)* gruel supplemented with fermented cowpea *(Vigna unguiculata)* flour and roasted melon seed *(Citrullus vulgaris)* paste

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This work examined the chemical and organoleptic profile of fermented maize gruel enriched with roasted melon and fermented local species of cowpea *(oraludi)*. The chemical composition of the processed food samples were assessed using standard methods. Blends were formulated in the ratio of 70: 30 (14 g protein basis) of maize gruel and cowpea flour or melon paste; and 70: 20: 10 (14 g protein basis) of maize gruel, cowpea flour and melon paste. Young students of the University community were selected for sensory evaluation. Sensory attributes were assessed with a nine-point Hedonic scale. Fermented maize gruel was the control. The proximate result reveals that melon paste had higher values than cowpea flour in protein, ash, crude fibre and fat (35.00: 29.75; 3.60: 2.40; 4.85: 2.33 and 39.95: 3.15%, respectively). Cowpea flour showed superior percentage value to melon paste in moisture and carbohydrate (8.76: 2.28; 57.26: 14.32%, respectively). Melon paste and cowpea flour had close values for iron (5.53: 5.48 mg/100 g, respectively) and zinc (0.019: 0.012 mg/100 g, respectively). There was not much difference in the phytate and tannins values of melon paste and cowpea flour (0.12: 0.18; 8.81: 10.48 mg/100 g, respectively). The blends were generally acceptable (5.46 to 6.88; p>0.05). Composite blends of locally available and under-utilized legumes should be used to add variety to infant diet.

Key words: Complementary food, gruel, chemical composition, organoleptic test.

INTRODUCTION

Problems associated with infant feeding include bulkiness and monotony of diet. Various processing techniques such as fermentation, dehulling, germination, drying and milling had been employed to combat the problems of bulkiness, acceptability, quality, flavour, texture, viscosity, palatability (Hotz and Gibson, 2007; Odunfa, 1985; Nnam, 2002). These methods create variety that eliminates monotony. The presence of anti-nutrients and food toxicants limit the full utilization of cereal-legume based infant foods. This calls for the exploitation of other nutrient dense foods as valid means of enriching infant gruels and promoting the health nutritional status of infants. Low protein content and quality and monotony of diet are still problems to mothers who cannot afford good protein sources. Commercial complementary foods modified to meet infant nutrient requirements are expensive and beyond most Nigerian families (Nwamarah and Amadi, 2009; Anigo et al., 2010). Hence many depend on inadequately processed traditional foods consisting mainly of unsupplemented cereal porridges made from

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Table 1. Ratios of composite blends formulation.

Code	Composite blends	Ratio	Gram
AK	Fermented maize (48 h)	100:0:0	319.6:0:0
LB	Fermented maize, fermented cowpea roasted (15 min)	70:30:0	223.75:7.75:0
CM	Fermented maize, fermented cowpea roasted (20 min)	70:30:0	223.75:7:0
GQ	Fermented maize: fermented cowpea roasted (15 min): roasted melon seed paste	70:20:10	223.75:5.15:2
PF	Fermented maize: fermented cowpea roasted (20 min): roasted melon seed paste	70:20:10	223.75:4.7:2
TJ	Fermented maize: roasted melon seed paste	70:0:30	223.75:0:6

maize, sorghum and millet (Nnam, 2002). These problems compromise the health, growth and development of infants thereby predisposing them to protein energy malnutrition (PEM) and infections (due to lowered immunity). PEM results when an infant's body need for energy and protein or both are not satisfied by their diets (Wardlaw and Hampl, 2007).

Most of our indigenous legumes which are cheap sources of plant protein are under exploited and rarely used for infant foods. Outside soybean and peanut and a few other legumes, the rest are consumed in only one or two forms. These varieties are equally cheap. They are cultivated in home gardens during planting seasons, therefore, are available. Melon seed *(Citrullus vulgaris)* is underutilized. Outside its use in soup preparation (*egusi* soup), only a few localities roast and eat it as snack and a few local recipes. The under-utilized local crops could be explored as cheap alternative and nutritionally adequate complementary foods.

MATERIALS AND METHODS

Yellow maize *(Zea mays),* dehulled melon *(Citrullus vulgaris)* seeds and a local variety of cowpea *(Vigna unguiculata;* local name: *oraludi)* used for this work were purchased from Nsukka main market in Enugu State, Nigeria.

Sample preparation

Six kilograms of maize grains was picked clean, washed and soaked in water in the ratio of 1: 3 w/v for 48 h. Thereafter, it was washed and wet-milled with water into slurry which was sieved using muslin cloth to remove husk. The filtrate was poured into a cotton bag and squeezed to remove excess water. Twenty gram (20 g) of the sample was used for chemical analysis while the remaining was poured into a polyethylene bag and stored in a freezer until needed.

Two hundred grams of dehusked melon seeds were washed with clean water and par boiled for 10 min such that the water dried up after cooking (to prevent loss of nutrients in the cooking water). The seeds were dried in a hot air oven (Model No 320, Gallenkamp, England) 80°C for 30 min; roasted for 15 min, and milled into fine paste using electric grinder. Twenty gram (20 g) of the sample was taken for chemical analysis while the remaining portion was stored in polyethylene bag and refrigerated until needed.

Five hundred grams of cowpea grains were steeped in water at room temperature and dehulled manually by rubbing them between palms (attrition). The dehulled grains were fermented (liquid state fermentation) for 24 h. It was divided into 2 portions and roasted for 15 and 20 min, respectively. Each portion was hammer milled separately (Model ED-5 Thomas Wiley, England) into fine flour (70 mm mesh screen) and stored in separate labelled polyethylene bags at room temperature until used.

Formulation of composites

The crude protein of each sample was estimated by the micro-Kjedahl method (Pearson, 1976). The composites were formulated on 14 g protein basis in the ratio of 100: 0: 0, 70: 30: 0 and 70:20:10 of fermented maize, fermented cowpea and parboiled roasted melon seeds. Six composites blends were formulated as follows (Table 1):

Chemical analysis

Association of Analytical chemists (AOAC, 1990) methods were used for chemical analyses. Crude protein was determined by micro- Kjeldahl method; crude fibre by acid hydrolysis; fat by soxhlet extraction method; ash by dry ashing method while carbohydrate was determined by difference. Minerals were evaluated using atomic absorption sphectrophotometric method; phytate by Harland and Oberleas (1986) and tannins by Price and Butler (1977).

Quantitative ration of composite blend

The gram portion of each composite was determined using simple proportion. The quantity of protein that should be supplied by each composite sample was determined from the ratio of composite blend (using 14 g protein as 100%). The quantity needed to fill each requirement was then derived using simple proportion (based on protein composition of the samples). The quantities derived were later halved because of the large quantities of sample that would not be required.

Preparation of gruel

For each of the products, the following recipe was used. For the 5 samples, 224.0 g of fermented maize paste (base) was dissolved in 50 ml of water (at room temperature) to form a slurry. Nine hundred millilitres of boiling water was added to the slurry while stirring until it gels (base). To each of the base, appropriate quantity of composite and 5 g of granulated sugar were added and stirred until well distributed. The samples were allowed to cool to 40°C (serving temperature) and separately kept in thermos flask to maintain the serving temperature for sensory evaluation.

Table 2. Proximate composition of fermented maize, fermented cowpea flours and roasted melon seed paste (%/100 g).

Composite	Moisture (%)	Protein (%)	Ash (%)	Crude fibre (%)	Fat (%)	CHO (%)
AK	46.00±0.05	2.19±0.02	1.50±0.05	0.10±0.02	1.60±0.05	48.61±0.01
FCR_1	8.76±0.06	27.13±0.15	2.30±0.6	2.25±0.06	2.30±0.06	57.26±0.93
FCR_2	8.48±0.05	29.75±0.1	2.40±0.51	2.33±0.05	3.15±0.08	53.89±0.79
PER	2.22±0.02	35.00±0.01	3.60±0.01	4.85±0.0	39.95±0.04	14.32±0.04

Mean±SD of 2 determinations; CHO, Carbohydrate; AK, Fermented maize (48 h); FCR_1, Fermented cowpea, roasted 15 min (flour); FCR_2 -, Fermented cowpea, roasted 20 min (flour) paste; PER, 10 min parboiled melon, roasted 15 min (paste).

Table 3. Iron and zinc content of fermented maize, fermented cowpea flours and roasted melon seed paste (mg/100 g dry weight basis).

Mineral	AK	FCR_1	FCR_2	PER
Iron (mg/100 g)	3.63±0.04	4.83±0.02	5.48±0.1	5.53±0.15
Zinc (mg/100 g)	0.07±0.05	0.01±0.01	0.01±0.01	0.12±0.08

Mean±SD of 2 determinations; AK, Fermented maize (48 h) paste; FCR_1, Fermented cowpea, roasted 15 min (flour); FCR_2 -, Fermented cowpea, roasted 20 min (flour); PER, 10 min parboiled melon, roasted 15 min (paste).

Sensory evaluation

Thirty students were randomly selected by balloting from third and final year students of the Department of Home Science, Nutrition and Dietetics, University of Nigeria, Nsukka. It was based on their previous participation in similar works. A nine-point Hedonic scale (Williams, 1981) was used to test for flavour, colour, texture and general acceptability of the gruels. The degree to which the product was liked was expressed as: like extremely (nine points), like very much (eight points), like moderately (seven points), like slightly (six points), neither like nor dislike (five points), dislike slightly (four points), dislike moderately (three points), dislike very much (two points), dislike extremely (one point). Like extremely to like slightly constituted good while dislike slightly to dislike extremely constituted poor. Neither like nor dislike showed that the product was neither good nor bad.

The sensory assessment was carried out in the Food Research Laboratory of Department of Home Science, Nutrition and Dietetics, University of Nigeria, Nsukka. The laboratory was adequately lit and free from distraction. The judges were arranged in such a way that they could not see the grading of each other. The samples were presented in plain coloured bowls and each judge was provided with a teaspoon and a glass of water to rinse his/her mouth after each testing. The testing was done around 11.00 am and the samples were presented at 40°C (serving temperature) in portions of 250 ml.

Statistical analysis

Means, analysis of variance (ANOVA) and Duncan's New Multiple Range Test (DNMRT) were the statistical tools employed. Significance was accepted at $P \leq 0.05$.

RESULTS

Chemical composition of fermented maize paste, fermented cowpea flours and roasted melon seed paste is presented in Table 2. The protein level of the samples ranged from 2.19% in fermented maize to 35.0% in melon seed paste. The ash values ranged from 1.5% in fermented maize paste to 3.6% in roasted melon seed paste. Cowpea flours (2.3 and 2.4%) and melon seed paste (3.6%) also proved superior to maize (1.5%) in terms of ash values. Roasted melon seed paste had high fat value (39.95%) as against fermented maize and cowpea flours whose values ranged from 1.6 to 3.15%. Fermented cowpea flours had the highest level of carbohydrate value (57.26 and 53.89%). This was followed by fermented maize (48.61%) while melon seed paste recorded the least carbohydrate value (14.32%).

Table 3 shows the iron and zinc content of the samples. The iron content of the samples ranged from 3.63 to 5.53 mg/100 g. PER recorded the highest value of 5.53 mg/100 g followed closely by FCR_2 (5.48 mg/100 g). The least value was found in AK (3.63 mg/100 g). Zinc content of the samples was low (0.01 to 0.12 mg/100 g). PER recorded the highest value of 0.12 mg/100 g followed by 0.07 mg/100 g in AK. The least value of 0.01 mg/100 g was recorded by both FCR_1 and FCR_2

Table 4 reveals the tannin, phytate and phytate zinc molar ratio of the samples. The level of tannins ranged from 5.60 to 11.48 mg/100 g. FCR_1 had the highest value of 11.48 mg followed by FCr_2 (9.96 mg/100g). The least value was recorded by AK (5.60 mg/100 g). The PZMR of the samples ranged from 0.28 to 1.76. FCR_1 had the highest value of 1.76 followed by FCR_2 with 0.66.

Table 5 shows the sensory attributes of the composite blends. The flavour of the blends in the Hedonic scale differed. It ranged from 5.20 to 6.83. GQ had the highest score (6.83) followed by TJ (6.27). However, the flavour difference observed in these gruels was not significant ($P>0.05$). In terms of colour, differences were observed. It

Table 4. Tannin, phytate and phytate zinc molar ratio (PZMR) of fermented maize paste, fermented cowpea flour and roasted melon seed paste in mg/100g dry weight basis.

Phytochemical	AK	FCR_1	FCR_2	PER
Tannin (mg/100g)	5.60	11.48	9.96	8.81
Phytate (mg/100g)	0.20	0.16	0.08	0.12
PZMR	0.28	1.76	0.66	0.63

Mean±SD of 2 determinations; AK; Fermented maize (48 h) paste; FCR_1, Fermented cowpea, roasted 15 min (flour); FCR_2 -, Fermented cowpea, roasted 20 min (flour); PER, 10 min parboiled melon, roasted 15 min (paste); PZMR, Phytate zinc molar ratio.

Table 5. Sensory evaluation of traditional fermented maize gruel enriched with either fermented cowpea flour and/or roasted melon seed paste (as consumed).

Composite	Flavour	Colour	Texture	General acceptability
AK	6.00 ± 0.34^a	7.20 ± 0.23^a	6.93 ± 0.32^a	6.29 ± 0.4^a
LB	5.20 ± 0.4^a	6.10 ± 0.27^a	6.57 ± 0.3^a	5.46 ± 0.4^a
CM	5.80 ± 0.39^a	6.90 ± 0.23^a	6.80 ± 0.31^a	5.88 ± 0.45^a
GQ	6.83 ± 0.29^a	7.37 ± 0.27^a	7.30 ± 0.23^a	6.88 ± 0.28^a
PF	6.20 ± 0.32^a	6.87 ± 0.28^a	7.03 ± 0.23^a	6.63 ± 0.35^a
TJ	6.27 ± 0.37^a	6.07 ± 0.37^a	7.00 ± 0.32^a	6.78 ± 0.31^a

Figures with the same subscript in the same column are not significantly different (P>0.05); Mean ± SEM of 30 respondents.

ranged from 6.07 to 7.37. GQ had the highest value (7.37) followed by AK (7.20) and TJ had the least (6.07). However, there was no significant difference in the colour attribute (P>0.05).

Texture of the gruels revealed some differences in the scores. GQ had the highest score (7.30), followed by PF (7.03) while LB had the least score (6.57). The differences, however, were not significant (P>0.05). In over all acceptability score of the gruels, the values differed. It ranged from 5.46 to 6.88. GQ had the highest score of 6.88, followed by TJ with 6.78 and PF with 6.63. In any case, no significant difference was observed (P>0.05).

DISCUSSION

The proximate composition of the samples collaborated with the report of other findings (Onoja and Obizoba, 2009; FAO, 2012). The low level of protein as observed in the fermented maize was because it is cereal. Cereals are not good sources of protein but legumes are (Ene-Obong, 2001) as can be seen in the high protein content of the cowpea and melon seed, in comparison. The high nutrient profile of roasted melon seed paste was attributed to its low moisture value. Its high ash value suggests a rich mineral source. The low ash value of maize implies that infants fed on maize gruel alone are at risk of micronutrient deficiency especially iron, zinc and calcium. The low fibre value of cowpea and maize was as a result of dehulling which not only rids the sample of its seed coat but also its vitamin B compounds. The least carbohydrate content of melon was attributed to its low moisture content. The higher the moisture values of food, the lower its nutrients per 100 g (Okeke and Obizoba, 1986). The high level of iron in the roasted melon seed paste implies a good complementary source to breast milk. Breast milk is a poor source of iron and as an infant grows older, its iron need is increased and stores depleted (Ene-Obong, 2001; Fraser et al., 2006). Roasted melon paste added to complementary food forms a good source of iron and absorption can be enhanced by introducing fruits in season. In addition, zinc status is enhanced, diarrhoea prevented and childhood infections curbed. Zinc is immune system booster (Wardlaw and Hampl, 2007). The low phytate zinc molar ratio (<5) of the samples infers good absorption of the zinc in the samples.

The similarity (P>0.05) in the sensory attributes of the samples suggests high acceptance and variety. The high general acceptability scores of the gruels was not a surprise. All had high scores for flavour, colour and texture and these were not significantly different (P>0.05). Fermentation and roasting add flavour to food and these may have influenced the general acceptability of the gruels. Onoja and Obizoba (2009) also reported high acceptability of 24 h fermented blends as a result of improved flavour and mutual supplementation.

Conclusion

Variety and adequacy are factors of importance in the formulation of complementary foods for children. The addition of roasted melon seed to maize and cowpea gruels proved more nutritious and better accepted. It should therefore, be incorporated into complementary mixes.

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The role of alpha and beta adrenergic receptors in cortisol-induced hyperglycaemia in the common African toad (*Bufo regularis*)

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The role of adrenergic receptors in cortisol-induced hyperglycaemia is not well known. The present study investigates the effects of adrenergic receptor blockers in cortisol-induced hyperglycaemia in the common African toad (*Bufo regularis*). Each toad was fasted and anesthetized with sodium pentobarbitone (3 mg/100 g i.p). The animals (control) received intravenous (i.v) injection of 0.7% amphibian saline while animals (untreated) were given cortisol (20 µg/kg). In pre-treatment groups, animals received prazosin (0.2 mg/kg i.v), propranolol 0.5 mg/kg or combination of prazosin (0.2 mg/kg i.v) and propranolol (0.5 mg/kg i.v) before i.v injection of cortisol (20 µg/kg). Thereafter, blood samples were collected for estimation of blood glucose level using the modified glucose oxidase method. Cortisol caused significant increase in blood glucose level from 44.4±3.8 to 71.7±9.7 mg/dl. Pre-treatment of the toads with propranolol (0.5 mg/kg i.v) caused significant reduction (p≤ 0.01) in cortisol-induced hyperglycaemia while pre-treatment with prazosin (0.2 mg/kg i.v) produced no significant effect on hyperglycaemia induced by cortisol. The combination of both prazosin and propranolol completely abolished the effects of cortisol on blood glucose level. The results suggest that cortisol-induced hyperglycaemia in the toad (*B. regularis*) is mediated probably by both the α- and β-adrenergic receptors with the beta adrenergic receptors playing dominant role.

Key words: Cortisol, hyperglycaemia, prazosin, propranolol, amphibian saline, common African toad.

INTRODUCTION

Cortisol is a well known hyperglycaemic agent. The hyperglycaemic effect has been reported in frogs (Hanke, 1974, 1978; Broughton and Deroos, 1984) and other animal species (Chan and Woo, 1978a; Leach and Taylor, 1982; Pretty et al., 2009). Its hyperglycaemic action is due to activation of gluconeogenesis (Baxter, 1979; Renaud and Moon, 1980; Khani and Tayek, 2001). The hyperglycaemic response to cortisol involves metabolic actions such as glucose release from the liver as a product of glycogenolysis, increase in gluconeo-genesis and decrease in peripheral glucose utilisation. The relative contribution of each of these effects differ in different species and under different nutritional conditions (Hanke,1978).The hyperglycaemia develops slowly, suggesting that protein catabolism and enzyme activation are necessary before glucose release. Corticosteroids interact with the sympathoadrenal system to enhance the actions of catecholamines and affect glucose metabolism and insulin sensitivity through a signaling pathway involving or intersecting with catecholamines and the sympathetic nervous system (Pretty et al., 2009). Previous studies in rats show that adrenal glucocorticoids facilitated beta receptors function *in vivo* and that adrenalectomy resulted in loss of responsiveness to

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to catecholamines (Davies et al., 1981; Davies and Lefkowtiz, 1984; Taylor and Hancox, 2000).The study of Yang and Zhang (2004) shows that corticosteroid hormones have permissive effect to the catecholamine through glucocorticoid receptors. Adrenergic receptors transmit adrenaline and noradrenaline signals between cells (Gilsbach and Hein, 2008) and are important mediators of physiologic responses to endogenous catecholamines (Gilsbach and Hein, 2012). Previous studies show also that there is interaction between the hormones adrenaline and cortisol in their physiologic actions (Peter, 2011; George et al., 2013). However, while the receptors mediating the hyperglycaemic response to other hormones like adrenaline and other sympathomimetics amines have been studied, the role of adrenergic receptors in cortisol-induced hyperglycaemia in amphibians has not received much attention. The aim of the present study was to find out the possible role of alpha and beta adrenergic receptors in cortisol-induced hyperglycaemic in the common African toad (*Bufo regularis*).

MATERIALS AND METHODS

Experiments were carried out on 240 adult toads (*B. regularis*) of both sexes weighing between 70-100 g. The toads were obtained from the banks of slow-moving streams, around ponds and wet bushes. The collection process is that of randomly picking the toads as one finds them during the night search. Hence selection of the animal is unbiased. Each animal was fasted 24 h before the start of the experiment and anaesthesized with sodium pentobarbitone 3 mg/100 g body weight given intraperitoneally. The animal was secured on its back on a dissecting board. The truncus arteriosus was dissected free from surrounding connective tissue and used for blood collection. The anterior abdominal vein was cannulated for drug injection. Each toad was heparinised (170 units/0.1 ml) and allowed 30 min stabilization. After stabilization period, basal blood collection (0 min) was made from the truncus arteriosus.

The animals were randomly divided into five groups (1-V) of 48 toads per group. Toads in group I (control) received intravenous (i.v) injection of 0.7 % amphibian saline while toads in group II (untreated) received cortisol (20 µg/kg i.v). Toads in groups III, IV, and V were pre-treated with prazosin (0.2 mg/kg i.v), propranolol (0.5 mg/kg i.v), or combination prazosin (0. mg/kg i.v) and propranolol (0.5 mg/kg i.v), respectively, 30 min before injection of cortisol (20 µg/kg i.v). In each animal, 0.05 ml per sample was drawn directly from the truncus arteriousus for glucose determination. Blood samples were collected at time interval of 0, 5, 10, 20, 30, 60, and 90 min, post-injection. Each drug injection was in a total volume between 0.1 and 0.12 ml given intravenously through the anterior abdominal vein cannula. Blood glucose was determined immediately using modified glucose oxidase method of Trinder (1969). Because of the small size of the toad, animals were sampled only once in each experiment and then sacrificed.

Determination of liver and muscle glycogen

To determine the glycogen content of *B. regularis*, six toads were collected and used per group. After surgical procedure and 30 min stabilization period, each animal was given 0.7% amphibian saline (control group) through anterior abdominal vein cannula. For the cortisol group, each animal received 20 µg/kg cortisol injection. In pre-treatment groups, animals received either prazosin (0.2 mg/kg i.v), (propranolol 0.5 mg/kg i.v) or combination of prazosin (0.2 mg/kg i.v) and propranolol (0.5 mg/kg i.v) before i.v injection of cortisol (20µg/kg). Thereafter, the whole liver and gastrocnemius muscle of each animal were removed quickly, 60 min post injection under anaesthesia, cleared of adherent tissues and blood was blotted away using blotting paper. They were weighed separately using an electronic weighing balance, model DT 1000 England. Immediately, one gram each of the liver and muscle of the toad was removed separately and the glycogen content determined using anthrone reagents method (Seifter et al., 1950; Jermyn, 1975).

Isolation and purification of glycogen

1 g of the liver and 1 g of the muscle each were placed in individual pre-heated Erlnmeyer flasks containing 10 ml of 30% KOH solution. The liver and muscle were digested separately by heating the flasks for 20 min in a steam bath with occasional shaking until the tissues dissolved. The solution was allowed to cool. Then, 4 ml of the aliquot from each of the flasks was taken and placed in a 15 ml centrifuge tube each. 5 ml of 95% ethanol was added to each sample, mixed and centrifuged for 5 min; it was then decanted and drained for 5 min. The glycogen precipitated from each sample was dissolved in 0.5 ml distilled H_2O separately and mixed thoroughly. This was reprecipitated with 5 ml of 95% ethanol and recovered by centrifugation. The centrifugation was repeated four times until a white precipitate was obtained. The final glycogen precipitate was dissolved in 2 ml of distilled H_2O. 0.5 ml aliquot was taken from the unknown glycogen solution obtained from above. Then, 0.5 ml of concentrated HCl, followed by 0.5 ml formic acid (88%) and 4 ml of anthrone reagent were added in a step wise manner. The anthrone reagent was added slowly and mixed thoroughly. 0.5 ml of distilled water was treated as above and used as a blank. Several dilutions of the glycogen standard (0.2 mg/ml) were prepared. The dilutions used were 0.1, 0.2, 0.3, 0.4 ml of standard glycogen solution with enough distilled water to make 0.5 ml. These dilutions of glycogen standard were then treated as above. A standard curve was prepared from this.

All the tubes containing the solutions were heated in boiling water for ten minutes and allowed to cool. A portion of the contents from each tube was poured into a cuvette, bubbles were allowed to disperse and the absorbance was read. The absorbance was read at 630 nm against the blank. Calculation of glycogen was done using Equation 1:

$$\text{Mg glycogen/100g fresh liver} = \text{Mg glycogen/ml} \times \frac{10}{4} \times \frac{2}{0.5} \times \frac{100}{\text{Total fresh liver weight}}$$

Statistical analysis

All values given are mean ±S.E.M of the variables measured. Values between two groups were compared using student T-test while One-way analysis of variance (ANOVA) was used to compare mean values in multiple groups.

RESULTS AND DISCUSSION

Effects of 0.7% saline and cortisol on blood glucose and glycogen content (liver and muscle)

Infusion of 0.7% amphibian saline had no effect on blood glucose level (Figures 1 and 2). The mean fasting glucose level in the toad, *B. regularis*, was 44.4±3.8 mg/dl. Infusion of cortisol 20 µg/kg caused significant

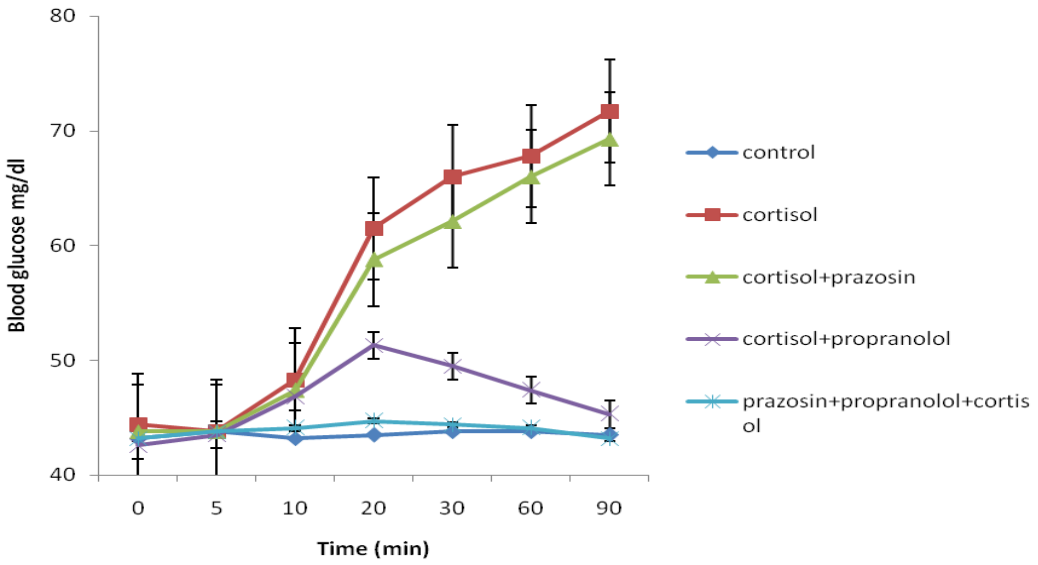

Figure 1. Effects of 0.7% amphibian saline cortisol (20 µg/kg) in untreated toads and in prazosin treated (0.2 mg/kg), propranolol treated (0.5 mg/kg), and both prazosin (0.2 mg/kg) and propranolol (0.5 mg/kg) on blood glucose levels of the treated groups. The points are mean±S.E.M. of seven determinations.

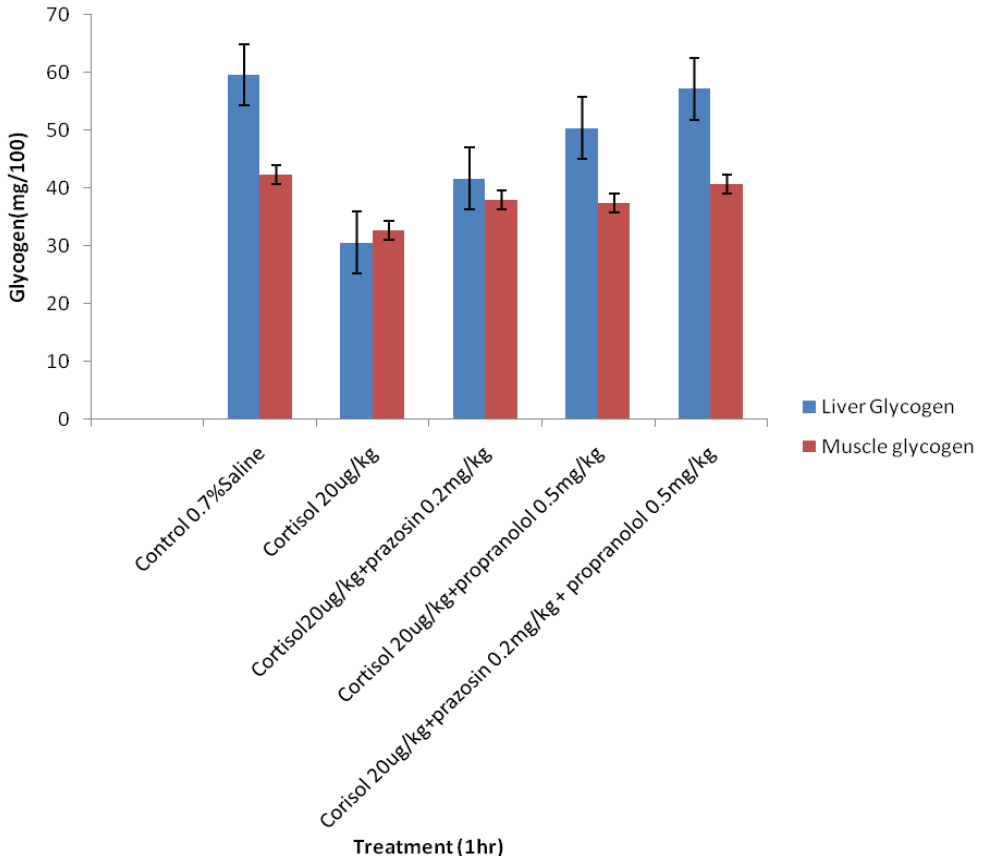

Figure 2. Graph of liver and muscle glycogen content (Mg glycogen/100 g fresh liver and muscle (mean±S.E.M) in toads infused with 0.7% amphibian saline, cortisol, and pre-treated with prazosin and propranolol.

increase in blood glucose level from a mean basal value of 44.4±3.8 mg/dl to maximum value of 71.7±9.7 mg/dl 90 min post injection and decrease in glycogen content of liver and muscle when compared with the control (Figures 1 and 2). Cortisol-induced hyperglycaemia was delayed and became significant 20 min post-injection period (Figure 1) and hyperglycaemia was progressive throughout the post- injection period.

Effects of pre-treatment with prazosin (0.2 mg/kg) and propranolol (0.5 mg/kg)

These are shown in Figure 1. The hyperglycaemic response to cortisol infusion was completely abolished by propranolol but prazosin did not produce any significant effect in cortisol-induced hyperglycaemia when compared with the untreated toads (Figure 1). A combination of both blockers completely abolished cortisol- induced hyperglycaemia in *B. regularis* when compared with the untreated toads (Figure 1). Figure 2 shows the effect of adrenergic blockers on liver and muscle glycogen. Pre-treatment of the toads with prazosin caused significant reduction in liver glycogen while pre-treatment with propranolol produced no significant reduction in liver and muscle glycogen level. The combination of both prazosin and propranolol caused non-significant reduction in liver and muscle glycogen (Figure 2).

The rise in blood glucose following cortisol injection in this study confirms its hyperglycaemic effect. The results agree with findings in frogs (Hanke, 1974, 1978; Broughton and DeRoos, 1984; Tavoni et al., 2013) and other animal species (Chan and Woo, 1978a; Khani and Tayek, 2001). The significantly higher levels of blood glucose following cortisol injection in the toad are probably due to an enhanced liver gluconeogenesis. Cortisol has been reported to exert its hyperglycaemic effect through activation of gluconeogenesis (Baxter, 1979; Renauid and Moon, 1980; Resmini et al., 2009). Al-Nagdy et al. (1995) reported increased levels of blood lactate and pyruvate following cortisone injection. In the present study, the stimulated liver gluconeogenesis may account for the decrease in liver and muscle glycogen following cortisol injection and compared with the control. Therefore, it is not unlikely that both the hepatic and muscle glycogen must have contributed some amount of glucose under the influence of cortisol. The results of the present study in which cortisol caused reduction in liver and muscle glycogen agrees with the findings in fishes (Foster and Moon, 1986; Vijayan and Leatherland, 1989) and in rats (Tavoni et al., 2013). Cortisol, a major glucocorticoid exerts its hyperglycaemic effect through activation of gluconeogenesis (Baxter, 1979; Renaud and Moon, 1980; Khani and Tayek, 2001). Since 0.7% amphibian saline injection had no effect on blood glucose, the hyperglycaemic effect of cortisol could not be ascribed to the stress of the injections.

In the prazosin pre-treated animals, the non-significant reduction in cortisol-induced hyperglycaemia seems to suggest that the alpha adrenergic receptors may not play a significant role in the production of hyperglycaemic response to cortisol in the toad. The significant reduction of liver glycogen by prazosin also, is an indication of the non significant involvement of alpha adrenoceptors in cortisol hyperglycaemia. The present findings contrast the report in man, that alpha adrenergic blockade by phentolamine infusion suppressed plasma adrenocorticotropic hormone (ACTH) and cortisol level while propranolol caused no significant change in plasma ACTH and cortisol although it enhanced plasma ACTH response to insulin induced hypoglycaemia (Nakai et al; 1973; Al-Damluji, 1988). Pretreatment with propranolol significantly reduced the rise in blood glucose response to cortisol injection. Since propranolol is a beta-blocker, it seems likely that the ability of cortisol to increase blood glucose in toads is mediated through the beta adrenergic receptors. Also, the non significant reduction in the liver and muscle glycogen in toads by propranolol further confirms that the beta adrenergic receptors are involved in the cortisol hyperglycaemia. The result is consistent with the study in rats in which glucocorticoids enhanced β-receptors mediated responses (Davies and Lefkowtig, 1984; Taylor and Hancox, 2000). Cortisol has been reported to exert its effects through the beta adrenergic receptors (George et al., 2013).The combination of both prazosin and propranolol completely abolished the rise in blood glucose following cortisol injection in the toad in the present study. Since prazosin alone did not cause a significant reduction in cortisol hyperglycaemia, the complete abolition of cortisol-induced hyperglycaemia by both prazosin and propranolol shows that prazosin potentiated the effect of propranolol on cortisol-induced hyperglycaemia. In conclusion, the results of the present study show that both the alpha and beta adrenergic receptors are involved in the cortisol-induced hyperglycaemia in the common African toad, *B. regularis*. However, the beta adrenergic receptors played a dominant role than the alpha adrenergic receptors in cortisol hyperglycaemia in the toad.

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