# African Journal of Biotechnology


Volume 14 Number 18, 6 May, 2015

ISSN 1684-5315

Academic Journals

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

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Examples:

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 Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc.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

Examples:

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

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

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

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Vol. 14(18), pp. 1525-1532, 6 May, 2015
DOI: 10.5897/AJB2015.14527
Article Number: 8AB382952706
ISSN 1684-5315
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http://www.academicjournals.org/AJB

African Journal of Biotechnology

*Review*

# A review on marine based nanoparticles and their potential applications

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Received 24 February, 2015; Accepted 22 April, 2015

**The increasing demands on nanoparticles have wide pertinent in almost all the fields. Marine ecosystem has variety of living resources, which includes prokaryotes like microorganism to eukaryotic organism like higher plants and animals. The present review dealt with the application of marine organisms in nanotechnology. Our discussion mainly focused on what the marine organisms are involved in and what type of nanoparticles is synthesized, including size and, medical and medicinal applications. Based on our observation through this review, it will be a good reference document for the further research on marine ecosystem to develop drug from sea.**

**Key words:** Nanomaterial, marine animals, mangroves, marine microbes.

## INTRODUCTION

In the recent years, biologically synthesized nanoparticles are of considerable interest in the area of biology and medicine due to their unique particle size and shape-dependence and their physical, chemical and biological properties (Ko et al., 2007). Most of the previous studies employed biomolecules (proteins, amino acids, carbohydrates and sugars), different type of whole cells of various microorganisms (bacteria, fungi and algae), or dissimilar plant resources (roots, leaves, flowers, bark powders, seeds, roots and fruits) for the synthesis of metal nanoparticles (Dahl et al., 2007; Kumar and Yadav, 2009; Huang et al., 2009; Laura et al., 2010). Marine organisms are rich source of bioactive compounds with remarkable impact in the field of pharmaceutical, industrial and biotechnological product developments. In recent years, the researchers focusing research on synthesis of nanoparticles from marine sources (Asmathunisha and Kathiresan, 2013) and as such they are both biocompatible and biodegradable which includes seashells, pearls and fish bones, and the particles ranged from 1 to 100 nm size. Biological entities from marine resources have typical nanostructures like diatoms and sponges are constructed with nanostructured cover of silica and coral reefs are with calcium which are arranged in significant architectures (Hoek et al., 1995). This review critically evaluates the existing knowledge on potential applications (Table 1) and current information about research on nanoparticles derived from marine organisms. This may help to fill the current knowledge gap and find exact remedy for serious problems.

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## MARINE FLORA-BASED NANOPARTICLES

Biosynthesis of nanoparticles by means of physical and chemical processes is highly expensive. In order to reduce the inevitable expenses in downstream processing of the synthesized nanomaterials and to increase the application of nanoparticles, the scientific community targeted the biological organisms. Nature has devised various processes for the synthesis of nano- and micro-length scaled inorganic materials which have contributed to the development of relatively new and largely unexplored area of research based on the biosynthesis of nanomaterials (Mohanpuria et al., 2008). Plants are important, safe and easily available source for nanoparticle synthesis with broad variability of metabolites that may aid in reduction. Numbers of plant are being currently investigated for nanoparticle synthesis for their efficacy and so many researches has been done with plants with respect to phytochemicals. The main phytochemicals responsible for their activity have been identified as terpenoids, flavones, ketones, aldehydes, amides and carboxylic acids (Prathna et al., 2010). In this regard, plants and plant part extracts based biosynthesis has been found to be cost effective and ecofriendly (Casida and Quistad, 2005). Environmental conditions of marine ecosystem and characterization of marine plants are extremely different from terrestrial ecosystem. Therefore, the marine plants might produce different types of bioactive compounds including polyphenols, flavonoids, alkaloids and tannins (Gnanadesigan et al., 2011a; Ravikumar et al., 2011a, 2011b; Ravikumar et al., 2010). Particularly, the biosynthesis of nanoparticles from mangroves and mangrove associates are very limited. Costal plants especially mangroves and mangrove associates are the good source of nanoparticles. In this respects, 26 costal flora were recorded for the production of silver nanoparticle by Asmathunisha (2010). An efficient and eco-friendly one-pot green synthesis of AgNPs using extracts of mangrove leaf buds has been reported (Umashankari et al., 2012).

In addition, the synthesized nanomaterials has number of applications as evidenced by the earlier reports in which antibacterial activity of silver nanoparticles from *Rhizophora mucronata* against marine ornamental fish pathogens such as *Proteus* spp., *Pseudomonas florescence* and *Flavobacterium* spp., isolated from an infected fish, *Dascyllus trimaculatus* (Umayaparvathi et al., 2013), and anti-cancer activity of silver nanoparticles from *Suaeda monoica* (Sathyavani et al., 2012) are reported. As per the preceding research biological synthesis of nanoparticles from the costal flora is a rich source for disease control.

## MARINE MICROORGANISM-BASED NANOPARTICLES

Microorganisms ranging from bacteria to fungi have been used in recent years as explained in detail in the Table 1 to develop non-toxic and environment friendly methods to synthesize nanoparticles (Bhattacharya and Rajinder, 2005). Some microorganisms can survive and grow even at high metal ion concentration due to their extraordinary resistant capability (Husseiny et al., 2007; Mohanpuria et al., 2007). Synthesis of nanoparticle using microbes offers better size control through compartmentalization in the periplasmic space and vesicles. The rate of intracellular particle formation and therefore, size of the nanoparticles could, to an extent, be manipulated by controlling parameters such as pH, temperature, substrate concentration and time of exposure to substrate (Gericke and Pinches, 2006). Marine microbes play several important roles in synthesis of nano-based drugs for human life improvement. Marine microbes have potential ability to synthesize nanoparticle for the reason that the marine microbes exist in the sea bottom, over millions of years in the past for reducing the vast amount of inorganic elements deep in the sea. Additionally, nanoparticles synthesized by microorganisms tend to be stabilized by peptides such as phytochelatins, thus preventing aggregation (Kang et al., 2008). These short peptides are synthesized in response to heavy metal stress and have been implicated as a universal mechanism to sequester metal ions in bacteria (Pages et al., 2008) and fungi (Guimaraes-Suares et al., 2007).

Nanotechnology involved in number of fields resulted in fulfilling the requirement of the human beings. In this the DNA, RNA and protein-based applications induced by nanotechnology are known as biomolecular nanotechhnology, the medical applications such as treatment and disease diagnosis are coming under the nanomedical technology (Sandhu, 2006). Many microorganisms are known to produce nanostructured particles with properties similar to chemically synthesized materials. This is the evidence documented earlier, formation of magnetic nanoparticles by magnetotactic bacteria, the production of silver nanoparticles within the periplasmic space of *Pseudomonas stutzeri* and the formation of palladium nanoparticles using sulphate reducing bacteria (Gericke and Pinches, 2006). Intracellular SNPs synthesized by a marine bacterium, *Idiomarina sp. PR58-8* which was found to be highly silver tolerant (Sachin Seshadri et al., 2012). The mangrove derived microbes *Escherichia coli*, *Aspergillus niger*, *Penicillium fellutanum* and Thraustochytrids capable of reducing the silver ions in faster rate with various antimicrobial applications (Kathiresan et al., 2009; Burja and Radianingtyas, 2005; Adams et al., 2006; Raghukumar, 2008; Gomathi, 2009; Kathiresan et al., 2010). Similar to this marine bacteria and fungi some of the mangrove-derived yeast species like *Pichia capsulata* and *Rhodosporidium diobovatum* also reported to have the nanoparticles synthesizing capacity (Manivannan et al., 2010; Seshadri et al., 2011). The marine cyanobacterium, *Oscillatoria willei* is known to

**Table 1.** Overview on nanoparticle biosynthesis by marine resources.

| Marine sources and year | Type of nanoparticle | Size (nm) | Name of the Species | Biological activity | Author |
|---|---|---|---|---|---|
| Mangroves | | | | | |
| 2013 | Silver | 4-26 | *Rhizophora mucronata* | Antimicrobial | Umayaparvathi et al., 2013 |
| 2012 | Silver | 71-110 | *Avicennia marina* (leaf, bark and root) | Antimicrobial | Gnanadesigan et al., 2012 |
| 2011 | Silver | 60-95 | *Rhizophora mucronata* | Larvicidal | Gnanadesigan et al., 2011a |
| 2011 | Silver | - | *Rhizophora apiculata* | Antibacterial | Antony et al., 2011 |
| 2010 | Silver | 5–20 | *Xylocarpus mekongensis* | Antimicrobial | Asmathunisha, 2010 |
| Coastal plant | | | | | |
| 2012 | Silver | 5-25 | *Prosopis chilensis* | Antibacterial to control vibriosis in *Penaeus monodon* | Kathiresan et al., 2013 |
| Salt marshes | | | | | |
| 2012 | Silver | 31 | *Suaeda monoica* | Anti-cancer | Satyavani et al., 2012 |
| 2010 | Silver | 50-90 | *Sesuvium portulacastrum* | Antimicrobial | Asmathunisha, 2010 |
| Sand dune | | | | | |
| 2012 | Silver | 85-100 | *Citrullus colosynthis* | Anti-cancer | Satyavani et al., 2011 |
| Algae | | | | | |
| 2014 | Silver<br>Gold | 2-17<br>2-19 | *Turbinaria conoides* | Antibiofilm activity | Vijayan et al., 2014 |
| 2014 | Silver | - | *Colpomenia sinuosa* | Anti-diabetic activity | Vishnu Kiran and Murugesan, 2014 |
| 2013 | Silver | 25-40 | *Padina gymnospora* | Antibacterial | Shiny et al., 2013 |
| 2013 | Silver | 45-76 | *Sargassum cinereum* | Antibacterial | Mohandass et al., 2013 |
| 2013 | Gold | 45-57 | *Gracilaria corticata* | Antimicrobial and antioxidant | Naveena and Prakash, 2013 |
| 2013 | Gold | 60 | *Turbinaria conoides* | Antibacterial | Rajeshkumar et al., 2013 |
| 2012 | Silver | 33-40 | *Sargassum ilicifolium* | Antibacterial and *in vitro* cytotoxicity | Kumar et al., 2012 |
| 2012 | Silver | 28-41 | *Ulva fasciata* | Antibacterial | Rajesh et al., 2012 |
| 2012 | Silver | 10-30 | *Ulva lactuca* | Antibacterial | Bharathiraja et al., 2012 |
| 2012 | Silver | 20-30 | *Urospora* sp. | Antibacterial | Suriya et al., 2012 |

**Table 1.** Contd.

| | | | | | |
|---|---|---|---|---|---|
| 2012 | Gold | 18.7-93.7 | *Stoechospermum marginatum* | Antibacteial | Arockiya et al., 2012 |
| 2012 | Silver | 10-72 | *Padina pavonica* | Microbicidal | Sahayaraj et al., 2012 |
| 2011 | Silver | 22 | *Gelidiella acerosa* | Antifungal | Vivek et al., 2011 |
| 2011 | Silver | 35 | *Gracilaria edulis* | - | Murugesan et al., 2011 |
| 2011 | Gold | 15-20 | *Laminaria japonica* | - | Ghodake and Lee, 2011 |
| 2007 | Gold | 8-12 | *Sargassum wightii* | - | Singaravelu et al., 2007 |
| Marine microbes (Cyanobacteria) | | | | | |
| 2013 | Silver | 44-79 | *Microcoleus* sp. | Antimicrobial | Sudha et al., 2013 |
| 2013 | gold | 60 | *Turbinaria conoides* | Antimicrobial | Rajeshkumar et al., 2013 |
| 2012 | Cadmium | 5 | *Phormidium tenue* | - | MubarakAli et al., 2012 |
| 2011 | Silver | 100-200 | *Oscillatoria willei* | - | MubarakAli et al., 2011 |
| 2008 | Silver<br>Gold<br>Biometallic | 7-16<br>6-10<br>17-25 | *Spirulina platensis* | - | Govindaraju et al., 2008 |
| Bacteria | | | | | |
| 2014 | Silver | 5-30 | *Shewanella algae* | Pest Control | Babu et al., 2014 |
| 2013 | Silver<br>Gold | 40-60<br>10-50 | *Stenottrophomonas* sp | - | Malhotra et al., 2013 |
| 2013 | Gold | 35-65 | *Klebsiella pneumoniae* | - | Malarkodi et al., 2013 |
| 2013 | Silver | 50-100 | *Vibrio alginolyticus* | - | Rajeshkumar et al., 2013 |
| 2013 | Silver | 42-94 | *Pseudomonas aeruginosa* | Antibacterial and Anti fungal | Rajeshkumar et al., 2013 |
| 2012 | Silver | 1-10 | *Pseudomonas fluorescens* | Antimicrobial | Prabhawathi et al., 2012 |
| 2012 | Gold | 10 | *Marinobacter pelagius* | - | Sharma et al., 2012 |
| 2012 | Silver | 25-50 | *Bacillus subtilis* | Anti fungal | Vijayaraghavan et al., 2012 |
| 2012 | Silver | 25 | *Idiomarina sp. PR58-8* | - | Seshadri et al., 2012 |
| 2011 | Silver | 20-100 | *Pseudomonas* sp. | - | Muthukannan and Karuppiah, 2011 |
| 2010 | Silver | 5-20 | *E. coli* | Antimicrobial | Kathiresan et al., 2010 |
| Fungi | | | | | |
| 2014 | Silver | 2-22 | *Aspergillus flavus* | - | Vala et al., 2014 |
| 2010 | Silver | 5-35 | *Aspergillus niger* | Antimicrobial | Kathiresan et al., 2010 |

**Table 1.** Contd.

| | | | | | |
|---|---|---|---|---|---|
| 2009 | Silver | 50-100 | *Thraustochytrium* sp. | - | Gomathi, 2009 |
| 2009 | Silver | 5-20 | *Penicillium fellutanum* | - | Kathiresan et al., 2009 |
| Yeast | | | | | |
| 2011 | Lead | 2-5 | *Rhodosporidium diobovatum* | - | Seshadri et al., 2011 |
| 2010 | Silver | 50-100 | *Pichia capsulata* | - | Manivannan et al., 2010 |
| 2009 | Gold | 7.5-23 | *Yarrowia lipolytica* NCIM 3589 | Cell-associated nanoparticle synthesis | Pimprikar et al., 2009 |
| Actinomycetes | | | | | |
| 2014 | Gold | 10-20 | *Streptomyces hygroscopicus* | - | Waghmare et al., 2014 |
| 2013 | Gold | 5-50 | *Streptomyces* sp | Antimalarial | Karthik et al., 2013 |
| Diatoms | | | | | |
| 2011 | Gold<br>Gold-Silica | 9-22 | *Navicula atomus, Diadesmis gallica* | - | Schrofel et al., 2011 |
| Marine animals | | | | | |
| 2013 | Silver | 10.5 | *Saccostrea cucullata* (Oyster) | Antimicrobial | Umayaparvathi et al., 2013 |
| 2010 | Gold | 7-20 | *Acanthella elongata* (Sponges) | - | Inbakandan et al., 2010 |
| 2009 | Silver | 5-10 | Cod liver oil (Fin fish) | - | Khanna and Nair, 2009 |

secrete the protein which is responsible for reduction of silver ions and stabilization of silver nanoparticles (Mubarak et al., 2011). Recent records of Mubarak et al. (2012) have reported the synthesis and characterization of cadmium sulphide (CdS) nanoparticles from the marine cyanobacterium, *Phormidium tenue* NTDM05.

## MARINE ALGAE-BASED NANOPARTICLES

Marine algae is widely used in food, medicine, and manufacturing industries (Chapman and Chapman, 1980; Yang, 2002) as explained in detail in the Table 1. It is a rich source of biologically active compounds, such as polysaccharides (alginate, laminaran, fucoidan), polyphenols, carotenoids, fiber, protein, vitamins and minerals (Kushnerova et al., 2010; Mizuno et al., 2009; Zyyagintsseva et al., 2003). The algal phytochemicals include hydroxyl, carboxyl, and amino functional groups, which can serve both as effective metal-reducing agents and as capping agents to provide a robust coating on the metal nanoparticles in a single step.

## MARINE ANIMALS-BASED NANOPARTICLES

Dolphins and whales have rough skin surface due to the presence of nanoridges. These ridges enclose a pore size of 0.2 $\mu m^2$ which is below the size of marine fouling organisms and hence there is no attachment of biofoulers(Kathiresan, 2007). Nano scaled structures found on shark skin and 'brick-and-mortar' arrangement like micro-architecture on nacre (mother of pearl) paved a way for the latest advances on production of synthetic designed materials, in particular to be used in

biomedicl applications (Luz and Mano, 2009; Dean and Bushan, 2010). An outline of findings on biosynthesis of nanoparticles from marine resources is presented in Table 1.

## MARINE BASED NANOPARTICLES ON INSECT/PEST MANAGEMENT

Crop loss to the turn of 30% in plants caused due to the insect pests infesting several crop plants. The use of chemical insecticides and pesticides in crop protection disturb the soil health, water bodies and finally it affects human health (Vinutha et al., 2013). The potential application and benefits of nanotechnology are enormous. Recently, Babu et al. (2014) synthesized silver nanoparticles from marine bacterium Shewanella algae to control pests. Nanotechnology in agriculture plays an important role in the slow release effects which includes pest control with increased shelf-life to various applications in the agricultural fields. More number of nanoparticles have been developed using marine organisms like plants, animals, microbes etc., for variety of application mentioned in the Table 1. But very few findings were reported for the insect pest management. It needs more attention for crop protection, to meet the satisfactory level of production and to increase our economic status of country. The agricultural application of nanotechnology can suggest development of efficient and potential implications for overcoming the management of pests in crops. Nanoparticles can be used in the formulations of pesticides, insecticides, insect repellents, pheromones and fertilizers (Barik et al., 2008).

## CONCLUSION

Synthesis of nanoparticle with the help of marine resources accomplishes the need for safe, stable and environment friendly particles since it involves diverse marine ecosystem that is freely available and moreover this biological synthesizing method does not involve harmful solvents and reduced downstream processing steps which shrink the cost for their synthesis. An important challenge in nanoparticle synthesizing technology is to tailor the properties of nanoparticles by controlling their size and shape. Using marine organisms and their bioactive substances, the biosynthesis of nanoparticles extra-cellularly would be constructive if it is produced in a controlled manner to their size and shape. Nanoparticles of desired size and shape have been obtained successfully using living organisms-simple unicellular organisms to highly complex eukaryotes. The marine ecosystem has captured a major attention in recent years, as they contain valuable resources that are yet to be explored much for the beneficial aspects of human life. The field of nano biotechnology is still in its infancy and more research needs to be focused on the mechanistics of nanoparticle formation from the marine resources which may lead to fine tune the process ultimately leading to the synthesis of nanoparticles with a strict control over the size and shape parameters. Therefore, it needs collaborative research of various disciplines to develop simple and cost-effective techniques to improve the quality of life.

## Conflict of Interests

The authors have not declared any conflict of interests.

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Vol. 14(18), pp. 1533-1542, 6 May, 2015
DOI: 10.5897/AJB2015.14478
Article Number: 9E756EA52708
ISSN 1684-5315
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http://www.academicjournals.org/AJB

African Journal of Biotechnology

*Full Length Research Paper*

# The use of multiplexed simple sequence repeat (SSR) markers for analysis of genetic diversity in African rice genotypes

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Received 5 February, 2015; Accepted 4 May, 2015

**Rice is an emerging food and cash crop in Eastern Africa. Thousands of germplasm accessions have been introduced from major rice breeding centers, such as the International Rice Research Institute (IRRI), and Africa Rice but the genetic variability among the introduced rice germplasm is unknown. Knowledge on genetic diversity would be useful in designing measures for comprehensive breeding and conservation. To address this knowledge gap, 10 highly polymorphic rice simple sequence repeat (SSR) markers were used to characterize 99 rice genotypes to determine their diversity and place them in their different population groups. The SSR markers were multiplexed in 3 panels to increase their throughput. An average of 15.9 alleles was detected, ranging from 6 alleles detected by marker RM7 to 30 by marker RM333. The UPGMA dendogram based on Nei's genetic distance cluster analysis, revealed 5 genetic groups among the genotypes tested. Analysis of molecular variance indicated that 97% of the diversity observed was explained by differences in the genotypes themselves, and only 3% was due to the sources from which the genotypes were obtained. This study sets the stage for further diversity analysis of all the available germplasm lines using SSR markers to ensure effective utilization and conservation of the germplasm.**

**Key words:** Genetic diversity, simple sequences repeat (SSR) markers, multiplexing, rice genotypes, structure.

## INTRODUCTION

Rice is an important food and commercial crop in Africa but in a country like Uganda, domestic consumption is higher than production (FAOSTAT, 2012). Uganda's rice cultivars (*Oryza sativa*, 2n = 24, AA) include NERICA lines, landraces, and varieties developed by the Cereals Program of Uganda's National Crops Resources Research Institute (NaCRRI). In an effort to identify the best and most diverse candidates with resistance to local stresses that may provide rapid genetic improvement and be incorporated into Uganda's national rice breeding efforts,

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NaCRRI breeders have imported thousands of rice lines from the International Rice Research Institute (IRRI) and AfricaRice center and evaluating them alongside locally-adapted lines and breeding materials. These available germplasm lines are morphologically diverse but lack adequate documented information on their genetic potential and diversity. This deters their apt utilization as a potential source of desired genes and their effective conservation for future use. Constraints to rice production in Uganda include both biotic and abiotic stresses, and these are frequently evolving. This requires genetically diverse materials to check genetic erosion resulting from the continued adoption of only particular varieties and thereby maintain and/or increase the region's rice production.

Genetic diversity in rice germplasm can be assessed by both observed morphological traits and molecular markers (Chakravarthi and Naravaneni, 2006). Though morphological traits have been used as markers for assessing genetic diversity in the past, they are often influenced by the environment, limited in number and are therefore unreliable in themselves (Miller et al., 1989). Different types of genetic (DNA) markers are available nowadays including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), restriction fragment length polymorphisms (RFLP), single nucleotide polymorphisms (SNP), simple sequence repeats (SSR) among others. Each marker type deferrers in cost, time requirement, degree of polymorphism detected, application and principle. SSRs are the preferred markers for genetic analysis in rice and have been used for a number of studies (Akagi et al., 1997) because of their abundance and well distribution throughout the genome. As genetic markers, they are co-dominant, detect high levels of allelic variation, and can be multiplexed to increase the throughput (Guichoux et al., 2011). They are also technically efficient, cost-effective and able to analyze both *indica* and *japonica* rice groups (McCouch et al., 2002).

Morishima and Oka (1981) divided the cultivated species of rice into two groups; *indica* and *japonica*. The domestication process was believed to have caused the difference between these two groups, including their reproductive barriers (Harushima et al., 2002). Furthermore, three morphological groups were described by ecological distribution; tropical *japonica*, temperate *japonica*, and *indica* (Glaszmannand Arraudeau, 1986). McCouch et al. (2002) developed a high-density rice genetic map from fully sequenced BAC (Bacterial Artificial Chromosome) and PAC (P1-derived Artificial Chromosome) clones representing 83% of the total rice genome BAC (Bacterial Artificial Chromosome) and PAC (P1-derived Artificial Chromosome). These consist of 2340 validated markers and the information was integrated into gramene, a comparative grass genome database (http://archive.gramene.org/markers/microsat/ssr.html) to increase the density and utility of the SSR map in rice (McCouch et al., 2002).

In this study, the utility of this vast genetic resource was evaluated by identifying SSR markers reliable for use in rapid molecular characterization of the collection of rice genotypes available in NaCRRI. As a pilot project in the program, only one marker was picked per rice chromosome basing on its degree of polymorphism as reported by other rice researchers (Drame et al., 2011; Chakravarthi and Naravaneni, 2006; Ni et al., 2002). This was aimed at detecting genetically diverse lines and classification into their different groups.

## MATERIALS AND METHODS

### Plant material

Ninety nine rice genotypes (Table 1) were used in this study. They included the local varieties; Supa, Kaiso, Sindano, NERICA 1, NERICA 2 and NERICA 10, and introductions from IRRI and AfricaRice, interspecific and intraspecific breeding lines from NaCRRI Cereals Program, and collections from Ugandan farmers. The study lines were selected on the basis of their phenotypic diversity observed in the field. They were planted in NaCRRI in central Uganda (located at 000 32' N latitude and 320 53' E longitude, and an altitude of 1,150 m asl). After one month of establishment, fresh young leaves were harvested from each genotype for DNA analysis.

### DNA extraction and quantification

The genomic DNA was extracted from about 100 mg of frozen leaf tissue at the Forestry and Agricultural Biotechnology Institute (FABI) of the University of Pretoria, South Africa using the Qiagen DNeasy plant kit (QIAGEN, 2006). Aliquots of 10 µl of the freshly extracted DNA were stained with Syber green and electrophoresed on 1% agarose gel then visualized under a UV transilluminator (BioRad) to assess their quality. The concentration of DNA in the samples was determined using a Nanodrop D-1000 spectrophotometer, and then diluted to 5 ng/µl prior to use (QIAGEN, 2006).

### Polymerase chain reaction (PCR)

The diluted DNA samples were amplified using 14 SSR markers (RM1, RM154, RM7, RM261, RM249, RM3, RM125, RM223, RM316, RM333, RM206, RM20A, RM273 and RM252) selected on the basis of their polymorphism level reported in rice by Chakravarthi et al. (2006) and Drame et al. (2011). The sequences of these molecular markers were obtained from the Gramene website (www.gramene.org/microsat/2013). The primers were synthesized by Inqaba Biotechnologies Inc (Pretoria, South Africa). PCR was done to confirm amplification and polymorphism of the markers in ten randomly selected DNA samples prior to labeling with fluorescent dyes. The PCR amplifications were carried out separately for each marker, in a 96-well DNA Engine Peltier Thermal Cycler (Biorad). The total volume of 10 µl PCR mix was constituted by 5 ng/µl DNA, 1x PCR buffer (Fermentas: 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgSO_4$, 1.5 mM $MgCl_2$), 2 mM dNTPs mix, 0.2 µM each of the forward and reverse primers, 25 mM $MgCl_2$, and 0.5U Taq polymerase (Fermentas). The PCR program used was 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. A final extension step at 72°C for 5 min was included. The markers such as RM1, RM261, RM3 and RM316 which were not as polymorphic as expected were not labelled.

**Table 1.** List of 99 genotypes used in the study and their alleles detected with 10 markers.

| No. | Genotype | Allele | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Origin | RM125 / Chr7 | RM249 / Chr5 | RM7 / Chr3 | RM206 / Chr11 | RM252 / Chr4 | RM273 / Chr4 | RM154 / Chr2 | RM20A / Chr12 | RM223 / Chr8 | RM333 / Chr10 |
| 1 | NERICA-L28 | AfricaRice | 122/122 | 121/121 | 182/182 | 166/166 | 243/243 | 210/210 | 168/168 | 231/257 | 157/157 | 174/177 |
| 2 | NERICA 1 X WAC 116 | NaCRRI | 125/125 | 121/121 | 174/174 | 168/178 | 251/253 | 200/200 | 165/148 | 210/231 | 157/157 | 185/189 |
| 3 | BGM 111 | IRRI | 125/125 | 117/117 | 168/168 | 184/184 | 195/195 | 200/200 | 189/194 | 228/231 | 147/157 | 177/177 |
| 4 | FAROX 503-3-11-F43-2-1 | AfricaRice | 125/125 | 119/119 | 168/168 | 132/132 | 195/195 | 200/204 | 181/201 | 210/228 | 157/157 | 185/189 |
| 5 | MET 121 | AfricaRice | 125/125 | 119/119 | 182/182 | 132/132 | 195/195 | 200/204 | 194/194 | 210/210 | 157/157 | 189/192 |
| 6 | K5 X NERICA 1 | NaCRRI | 122/122 | 117/121 | 182/182 | 132/132 | 195/195 | 200/200 | 176/194 | 225/228 | 157/157 | 189/191 |
| 7 | NERICA 1 X NERICA 4 | NaCRRI | 125/125 | 119/119 | 174/174 | 132/138 | 195/195 | 204/204 | 148/201 | 210//311 | 155/157 | 185/189 |
| 8 | IR-80353-63-B-38-1-2-B | IRRI | 122/125 | 119/119 | 182/182 | null | null | null | 181/201 | 228/228 | 157/157 | 174/174 |
| 9 | IR-77372-7-B-4-1-1-B | IRRI | 122/125 | 119/119 | 174/174 | 132/132 | 195/195 | 204/204 | 201/201 | 210/311 | 157/157 | 189/189 |
| 10 | IR-80352-4-B-24-2-3-B | IRRI | 125/125 | 119/119 | 174/174 | 190/192 | 195/195 | 202/202 | 148/203 | 210/210 | 147/155 | 155/155 |
| 11 | IR 83372-B-B-133-2 | IRRI | 148/148 | 117/121 | 182/182 | 168/168 | 195/195 | 200/200 | 148/208 | 210/231 | 147/157 | 177/177 |
| 12 | NM7-8-2-B-P-2-1 | NaCRRI | 125/125 | 145/145 | 168/174 | 132/132 | 249/251 | 200/200 | 181/201 | 210/276 | 157/157 | 185/189 |
| 13 | WAB 2099-WAC1-TGR5-B | AfricaRice | 122/122 | 119/119 | 182/182 | 153/153 | 257/259 | 200/200 | 144/158 | 212/212 | 155/158 | 124/193 |
| 14 | WAB 2060-3-FKR2-3-TGR5-B | AfricaRice | 125/125 | 119/123 | 174/174 | 190/192 | 195/195 | 200/200 | 155/194 | 210/239 | 155/155 | 185/189 |
| 15 | BGM-5 | IRRI | 148/148 | 121/121 | 182/182 | 170/170 | 247/249 | 210/210 | 148/189 | 216/257 | 157/157 | 183/183 |
| 16 | NERICA 4 X NERICA 6 | NaCRRI | 125/125 | 119/121 | 182/182 | 132/132 | 195/195 | 200/200 | 148/194 | 210/210 | 147/147 | 189/192 |
| 17 | NERICA 4 X K5 | NaCRRI | 122/125 | 119/121 | 182/182 | 132/146 | 195/195 | 204/204 | 148/201 | 210/257 | 157/157 | 189/189 |
| 18 | WAB 2151-TGR2-WAT B3 | AfricaRice | 122/122 | 121/121 | 182/182 | 132/132 | 195/195 | 204/210 | null | 210/257 | 157/157 | 220/248 |
| 19 | Sindano | Farmers | 125/125 | 121/121 | 176/176 | 199/199 | 195/195 | 202/202 | 157/183 | 210/311 | 157/157 | 212/209 |
| 20 | AER-41 | IRRI | 148/148 | 121/121 | 182/182 | 168/168 | 247/249 | 210/210 | 176/176 | 231/260 | 155/155 | 185/189 |
| 21 | NERICA 6 X NERICA 4 | NaCRRI | 125/125 | 119/123 | 174/174 | 190/192 | 195/195 | 200/200 | 181/194 | 210/228 | 155/157 | 174/189 |
| 22 | ART3-11L1P1-B-B-2 | IRRI | 125/125 | 119/119 | 174/174 | 132/132 | 195/195 | 204/204 | 201/201 | 210/210 | 157/157 | 189/189 |
| 23 | IR 84852-B-44-1AER | IRRI | 122/122 | 119/119 | 168/168 | 146/146 | 247/249 | 210/210 | 157/157 | 216/216 | 157/157 | 147/177 |
| 24 | Namche-5 | NaCRRI | 122/122 | 119/119 | 182/182 | 105/151 | 257/259 | 200/200 | 181/181 | 216/216 | 157/157 | 157/181 |
| 25 | WAC 117 | AfricaRice | 148/148 | 121/121 | 168/168 | 168/168 | 251/253 | 200/200 | 189/189 | 231/276 | 157/157 | 177/177 |
| 26 | NERICA 4 X WAC 116 | NaCRRI | 125/125 | 121/121 | 174/174 | 132/168 | 195/195 | 204/204 | 157/157 | 210/216 | 157/157 | 177/179 |
| 27 | NERICA 1 X GIGANTE | NaCRRI | 122/125 | 119/119 | 174/174 | 190/192 | 195/195 | 204/204 | 147/196 | 210/258 | 147/147 | 187/189 |
| 28 | WAC 117 X NERICA 4 | NaCRRI | null | null | null | 132/132 | 251/253 | 200/204 | 157/189 | 231/276 | 157/157 | 177/177 |
| 29 | NERICA 1 X NERICA 6 | NaCRRI | 122/125 | 119/119 | 182/182 | 151/151 | 195/195 | 204/204 | 157/181 | 210/257 | 157/157 | 155/177 |
| 30 | IR-80352-4-B-24-2-3-B | IRRI | 122/122 | 115/115 | 170/170 | 146/146 | 217/217 | 210/210 | 165/201 | 234/257 | 138/157 | 174/177 |
| 31 | NM7-5-2-B-P-79-7 | NaCRRI | 125/125 | 123/123 | 174/174 | 194/195 | 195/195 | 204/204 | 194/196 | 210/210 | 161/161 | 194/196 |
| 32 | WAC 116 | AfricaRice | 116/122 | 121/121 | 182/182 | 146/146 | 217/217 | 210/210 | 157/189 | 231/257 | 157/157 | 174/177 |
| 33 | AER-75 | IRRI | 144/148 | 121/121 | 168/168 | 168/168 | 251/253 | 200/200 | 189/189 | 231/276 | 165/165 | 174/177 |
| 34 | NERICA 1 X NERICA 6 | NaCRRI | 122/122 | 119/119 | 182/182 | 151/151 | 257/259 | 200/200 | 181/181 | 210/228 | 157/157 | 177/177 |
| 35 | BGM-78 | IRRI | 122/122 | 121/121 | 182/182 | 146/146 | 217/217 | 210/210 | 138/201 | 234/254 | 138/138 | 174/177 |
| 36 | MET 124 | AfricaRice | 125/125 | 117/117 | 174/174 | 146/146 | 259/261 | 200/200 | 176/176 | 234/257 | 157/157 | 183/183 |
| 37 | NM7-27-1-B-P-77-6 | NaCRRI | 122/122 | 121/121 | 182/182 | 178/178 | 195/195 | 202/202 | 147/196 | 210/304 | 147/147 | 185/189 |
| 38 | NERICA 1 X K85 | NaCRRI | 122/122 | 121/121 | 182/182 | 168/168 | 217/217 | 210/210 | 147/176 | 231/260 | 157/157 | 174/177 |
| 39 | NM7-29-4-B-P-80-8 | NaCRRI | 125/125 | 123/123 | 174/174 | 182/182 | 195/195 | 204/204 | 196/196 | 210/277 | 161/161 | 182/185 |

**Table 1.** Contd.

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 40 | AER-41 | IRRI | 122/122 | 119/119 | 170/170 | 146/146 | 247/249 | 210/210 | 148/201 | 216/257 | 157/157 | 177/177 |
| 41 | WAB95-B-B-40-HB | AfricaRice | 125/125 | 119/119 | 174/174 | 132/132 | 195/195 | 204/204 | 196/196 | 210/210 | 157/157 | 185/189 |
| 42 | GIGANTE X K85 | NaCRRI | 122/122 | 119/119 | 182/182 | 132/151 | 195/253 | 200/200 | 181/181 | 231/276 | 157/159 | 174/174 |
| 43 | ART3-11L1P2-B-B-2 | IRRI | 125/125 | 119/119 | 174/174 | 184/184 | 195/195 | 204/204 | 201/201 | 210/259 | 163/163 | 188/191 |
| 44 | WC-50 IR 77372-7 | IRRI | 122/122 | 119/119 | 170/170 | 126/126 | 217/217 | 210/210 | 168/168 | 228/231 | 157/157 | 174/177 |
| 45 | K85 X WAC116 | NaCRRI | 122/122 | 121/121 | 182/182 | 146/146 | 217/217 | 210/210 | 165/165 | 231/285 | 157/157 | 174/174 |
| 46 | WAB 2099-WAC1-TGR5-B | AfricaRice | 122/122 | 121/121 | 182/182 | null | null | 210/210 | 168/168 | 210/231 | 157/157 | 155/157 |
| 47 | IRAT325/WAB 450-B-136-HB | IRRI | 125/125 | 119/119 | 174/182 | 132/132 | 195/195 | 204/204 | 165/176 | 210/231 | 155/157 | 174/189 |
| 48 | WAB 2056-1-FKR4 | AfricaRice | 122/122 | 121/121 | 182/182 | 126/126 | 217/217 | 210/210 | 138/189 | 228/231 | 137/137 | 174/177 |
| 49 | K5 X GIGANTE | NaCRRI | 148/148 | 121/121 | 168/168 | 132/149 | 257/259 | 200/200 | 176/176 | 216/231 | 157/157 | 177/177 |
| 50 | NERICA 4 X GIGANTE | NaCRRI | 122/125 | 119/145 | 168/182 | 151/151 | 195/195 | 200/204 | 148/189 | 231/276 | 157/157 | 189/189 |
| 51 | Jaribu | AfricaRice | 112/122 | 121/121 | 182/182 | 160/160 | 217/217 | 210/210 | 148/189 | 210/260 | 157/157 | 177/177 |
| 52 | BGM-117 | IRRI | 112/122 | 121/121 | 182/182 | 126/126 | 253/255 | 200/200 | 148/201 | 216/260 | 157/157 | 177/177 |
| 53 | BGM IR 09L 223 | IRRI | 112/122 | 119/119 | 182/182 | 126/126 | 253/255 | 200/200 | 148/201 | 216/260 | 157/157 | 177/177 |
| 54 | BGM-111 | IRRI | 112/122 | 121/121 | 182/182 | 164/164 | 249/249 | 210/210 | 148/189 | 231/257 | 157/157 | 177/177 |
| 55 | NM7-27-1-B-P-77-6 | NaCRRI | 112/125 | 117/117 | 174/174 | 164/164 | 247/249 | 210/210 | 148/189 | 231/257 | 157/157 | 174/177 |
| 56 | WAB 1436-20N-3-B-FKR2-WAC1 | AfricaRice | 112/122 | 121/121 | 182/182 | 164/164 | 247/249 | 210/210 | 148/189 | 231/257 | 157/157 | 174/177 |
| 57 | NERICA 1 X WAC 117 | NaCRRI | 112/122 | 117/117 | 168/168 | 168/195 | 195/195 | 204/204 | 148/189 | 231/257 | 157/157 | 174/177 |
| 58 | NERICA 6 X K5 | NaCRRI | 125/125 | 119/119 | 174/174 | 138/146 | 195/195 | 202/202 | 148/196 | 210/239 | 155/155 | 191/194 |
| 59 | NERICA-L49 | AfricaRice | 112/122 | 121/147 | 182/182 | 146/146 | 217/217 | 210/210 | 168/168 | 231/257 | 159/159 | 159/174 |
| 60 | IR 77372-3-B-6-2-BB | IRRI | 122/125 | 119/119 | 174/174 | 132/132 | 195/195 | 204/204 | 148/201 | 210/210 | 157/157 | 185/189 |
| 61 | IR 83383-B-B-141-AER | IRRI | 112/122 | 121/121 | 182/182 | 166/166 | 247/249 | 210/210 | 189/189 | 231/257 | 157/157 | 180/182 |
| 62 | CEHIRANG | AfricaRice | 112/122 | 121/121 | 182/182 | 146/146 | 217/217 | 210/210 | 168/168 | 231/257 | 157/157 | 185/189 |
| 63 | TXD 306 (NaCRRI) | NaCRRI | 112/148 | 119/119 | 168/168 | 166/166 | 244/244 | 210/210 | 146/189 | 231/257 | 147/147 | 174/177 |
| 64 | WAC 116 | AfricaRice | 122/125 | 119/119 | 174/174 | 132/132 | 195/195 | 204/204 | 196/196 | 210/210 | 157/157 | 185/189 |
| 65 | NERICA-L49 | AfricaRice | 112/122 | 121/121 | 182/182 | 146/146 | 217/217 | 210/210 | 168/168 | 231/257 | 159/159 | 160/174 |
| 66 | IR 77372-3-B-6-2-BB | IRRI | 112/122 | 119/119 | 170/170 | 160/160 | 217/217 | 210/210 | 148/168 | 231/283 | 157/157 | 180/183 |
| 67 | NERICA-L50 | AfricaRice | 112/122 | 121/121 | 182/182 | 146/146 | 217/217 | 210/210 | 165/165 | 231/285 | 157/157 | 174/174 |
| 68 | AER-41 | IRRI | 112/122 | 119/119 | 170/170 | 146/146 | 249/251 | 210/210 | 148/201 | 216/257 | 157/157 | 174/177 |
| 69 | BGM-100 | IRRI | 112/122 | 121/121 | 170/170 | 146/146 | 217/217 | 210/210 | 168/168 | 234/257 | 155/155 | 174/177 |
| 70 | NERICA 1 X K85 | NaCRRI | 112/122 | 121/121 | 182/182 | 146/146 | 217/217 | 210/210 | 168/168 | 231/257 | 157/157 | 155/157 |
| 71 | NERICA 1 X K5 | NaCRRI | 122/125 | 119/119 | 182/182 | 132/132 | 195/195 | 204/204 | null | null | 149/160 | 236/236 |
| 72 | MET 113 | AfricaRice | 122/148 | 121/121 | 170/170 | 129/168 | 251/253 | 202/202 | 189/189 | 231/257 | 157/157 | 155/157 |
| 73 | IR 84852-B-44-1-4 AER | IRRI | 122/122 | 119/119 | 168/168 | 146/146 | 217/217 | 210/210 | 189/189 | 216/257 | 157/157 | 177/177 |
| 74 | K 85 | NaCRRI | 122/122 | 119/119 | 182/182 | 153/153 | 257/259 | 200/200 | 181/181 | 228/285 | 157/157 | 174/174 |
| 75 | NM7-8-2-B-P-2-1 | NaCRRI | 125/125 | 87/123 | 174/174 | null | null | 225/225 | 161/196 | 210/280 | 161/161 | 185/189 |
| 76 | IR-77372-7-B-4-1-1-B | IRRI | 122/122 | 119/121 | 182/182 | 146/168 | 217/257 | 200/210 | 165/181 | 228/231 | 157/157 | 174/180 |
| 77 | WAB 2101-WAC3-1-TGR1-WAT B7 | AfricaRice | 122/122 | 121/121 | 182/182 | 146/146 | 217/217 | 210/210 | 165/165 | 231/285 | 157/157 | 174/174 |
| 78 | BGM-5 | IRRI | 122/122 | 121/121 | 182/182 | 146/146 | 217/217 | 210/210 | 165/165 | 231/285 | 157/157 | 174/174 |
| 79 | GIGANTE | Farmers | null | 117/117 | null | 132/132 | 251/253 | 200/200 | 181/181 | 231/276 | 157/157 | 150/157 |
| 80 | Supa | Farmers | 122/122 | 121/121 | 170/170 | 168/168 | 251/253 | 210/210 | 176/176 | 237/263 | 147/147 | 183/185 |

**Table 1.** Contd.

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 81 | K 5 | NaCRRI | 122/122 | 119/119 | 182/182 | 146/146 | 217/217 | 210/210 | 168/168 | 231/257 | 157/157 | 174/177 |
| 82 | NERICA 1 X GIGANTE | NaCRRI | 122/125 | 121/121 | 182/182 | 132/132 | 195/195 | 204/204 | 181/181 | 210/210 | 157/157 | 155/157 |
| 83 | IR 79253-55-1-4-6AER | IRRI | 122/125 | 123/123 | 174/174 | 146/166 | 217/217 | 210/210 | 148/189 | 231/257 | 157/157 | 177/177 |
| 84 | NERICA 6 X NERICA 1 | NaCRRI | 122/122 | 119/119 | 182/182 | 190/192 | 195/195 | 200/200 | 148/196 | 210/239 | 147/155 | 185/189 |
| 85 | IR-80353-63-B-38-1-2-B | IRRI | 122/122 | 121/121 | 182/182 | 146/146 | 217/217 | 210/210 | 138/201 | 234/254 | 137/137 | 174/177 |
| 86 | IR 09N 505 | IRRI | 122/122 | 119/121 | 182/182 | 146/146 | 217/217 | 210/210 | 189/189 | 231/257 | 157/157 | 174/177 |
| 87 | NM7-9-2-B-P-2-1 | NaCRRI | 122/122 | 130/130 | 174/174 | 129/129 | 257/259 | 200/200 | 181/181 | 228/285 | 157/157 | 185/189 |
| 88 | IR 83372-B-B-133-2 | IRRI | 122/122 | 121/121 | 182/182 | 146/146 | 257/259 | 210/210 | 168/168 | 231/257 | 165/167 | 180/183 |
| 89 | IR 77372-3-B-6-2-BB | IRRI | 125/125 | 119/119 | 174/174 | 184/184 | 195/195 | 204/204 | 148/201 | 210/259 | 168/168 | 191/191 |
| 90 | FAROX 503-3-11-F43-2-1 | IRRI | 112/122 | 119/119 | 182/182 | 166/166 | 229/229 | 210/210 | 168/168 | 231/257 | 157/157 | 177/177 |
| 91 | MET 103 | AfricaRice | 125/125 | 130/130 | 176/176 | 197/199 | 195/195 | 204/204 | 183/183 | 210/210 | 157/157 | 185/185 |
| 92 | WAB 2151-TGR2-WAT B3 | AfricaRice | 122/122 | 121/121 | 182/182 | 146/146 | 217/217 | 210/210 | 168/168 | 231/257 | 159/159 | 157/174 |
| 93 | IR-77381-2-1-7-1-1-B | IRRI | null | 130/130 | 176/176 | 197/199 | 195/195 | 204/204 | 183/183 | 210/210 | 157/157 | 182/185 |
| 94 | SK-19-38-2 | AfricaRice | null | 130/130 | 170/170 | 129/129 | 157/159 | 200/200 | 176/176 | 234/257 | 153/153 | 180/180 |
| 95 | NM7-30-4-B-P-80-8 | NaCRRI | 125/125 | 119/119 | 174/174 | 132/132 | 195/195 | 204/204 | 148/196 | 210/210 | 157/157 | 185/189 |
| 96 | WAB2066-6-FKR4-WAC1-TGR1-B-WAT-B1 | AfricaRice | 122/122 | 121/121 | 182/182 | 146/146 | 217/217 | 210/210 | 165/165 | 231/285 | 157/157 | 174/174 |
| 97 | WAB 2076-WAC2-TGR1-B | AfricaRice | 122/122 | 121/121 | 182/182 | 146/146 | 217/217 | 210/210 | 165/165 | 210/231 | 157/157 | 171/174 |
| 98 | PVS-101 | IRRI | 122/122 | 121/121 | 182/182 | 146/146 | 217/217 | 210/210 | 165/165 | 231/285 | 157/157 | 174/174 |
| 99 | BGM-73 | IRRI | 148/148 | 121/121 | 168/168 | 126/126 | 247/247 | 210/210 | 148/189 | 210/210 | 167/167 | 174/177 |

Chr = Chromosome.

**Multiplexing and genotyping**

The forward primer for each of the remaining polymorphic markers was labeled at the 5' end with one of the following fluorescent dyes: 6-FAM (Blue), VIC (Green), NED (Yellow), and PET (Red) (Life technologies, USA). To increase the throughput of standard SSR analysis, which yields genotypic information at only one locus per reaction, multiplex PCR was done to boost genotyping by amplifying three or four loci in the same reaction (Guichoux et al., 2011). Primers that have the same annealing temperature were given different dye colors since the alleles they amplify overlap (Masi et al., 2003). These multiplexed primers were used together in the same PCR cocktail to make one panel (Table 2).

**Capillary electrophoresis and allele calling**

The MicroAmp plates were fed into a 24 capillary genetic analyzer 3500 GeneScan (Life Technologies, USA). Four injections into each 96-well plate, lasted 45 min each. The fragments generated were analyzed using GeneMapper V4.1 software (Life Technologies) to score exact lengths of the alleles (Applied Biosystems Inc. USA). A single peak was detected at a particular SSR locus for a homozygote or a pair of peaks for a heterozygote. The internal lane size standard GeneScan-500 LIZ was used to automatically calculate the fragment sizes that range from 35 to 500 base pairs. The size standard peaks corresponding to 35 to 90 base pairs were excluded from the analysis because of their proximity to primer peaks (Life Technologies, 2014). To reduce genotyping errors and increase the precision of allele sizing, the auto-bining method of GeneMapper V4.1 was used to create bins which represented mean sizes of alleles (in base pairs) of a particular allele category. These adjusted bins were embedded into GeneMapper V4.1 and were used to correct allele sizing (allele calling) when data was reanalyzed (Life Technologies, 2014).

**Data analysis**

The population structure of the samples/genotypes was determined using a model-based program, STRUCTURE V2.3.4 (Pritchard et al., 2000). The maximum number of populations for the simulation was set at 10 (K=10), the length of Burnin period at 5,000 and the number of Markov chain Monte Carlo (MCMC) repetitions after analysis equal at 50,000. The Admixture model of the program was used and allele frequencies were correlated. The limit for assigning a sample to a particular population was set at 75% genomic ancestry. The genetic distance between the genotypes was calculated with DARwin V5 software, using simple matching method. A phylogenetic tree was built using the Unrooted Weighted Neighbor-Joining (UWNJ) algorithm. Statistical parameters were set using GenAlEx 6.5 software Peakall and Smouse, 2006), to define molecular diversity, such as heterozygosity, fixation index, Shannon's information index and the analysis of molecular variance (AMOVA).

## RESULTS AND DISCUSSION

### Allelic diversity of the rice genotypes

The SSR markers used were able to determine

**Table 2.** Three multiplex panels consisting of 10 rice SSR markers were used in this study for diversity analysis. SSR marker allele sizes, fluorescently labeled dye, repeat motifs, chromosome no and annealing temperature information are mentioned.

| Panel | Marker | Annealing temp. (°C) | Chromosome no. | Allele size range (bp) | Dye color | Repeat motif |
|---|---|---|---|---|---|---|
| 1 | RM7 | 55 | 3 | 140-190 | VIC | (GA)n |
| | RM249 | 55 | 5 | 100-160 | NED | (GA)n |
| | RM125 | 62 | 7 | 100-150 | 6-FAM | (CTT)n |
| 2 | RM206 | 55 | 11 | 110-190 | VIC | (GA)n |
| | RM273 | 55 | 4 | 160-220 | NED | (TAC)n |
| | RM252 | 55 | 4 | 140-270 | 6-FAM | (TC)n |
| 3 | RM223 | 60 | 8 | 110-180 | VIC | (CT)n |
| | RM333 | 60 | 10 | 120-190 | NED | (TAT)n(CTT)n |
| | RM20A | 60 | 12 | 200-280 | 6-FAM | (TAA)n |
| | RM154 | 60 | 2 | 110-190 | PET | (CTT)n |

The labeled SSR markers were synthesized by Life Technologies Inc., USA.

**Table 3.** Estimated genetic diversity parameters obtained at each locus across 99 genotypes.

| Marker | No. of Alleles | He | Ho | I | F | Fst |
|---|---|---|---|---|---|---|
| RM125 | 7 | 0.554 | 0.216 | 0.950 | 0.616 | 0.120 |
| RM249 | 9 | 0.481 | 0.075 | 0.862 | 0.845 | 0.192 |
| RM7 | 6 | 0.600 | 0.013 | 1.055 | 0.980 | 0.094 |
| RM206 | 24 | 0.707 | 0.144 | 1.624 | 0.815 | 0.111 |
| RM252 | 16 | 0.659 | 0.212 | 1.378 | 0.701 | 0.141 |
| RM273 | 7 | 0.466 | 0.035 | 0.854 | 0.928 | 0.167 |
| RM154 | 21 | 0.809 | 0.412 | 1.928 | 0.474 | 0.074 |
| RM20A | 23 | 0.769 | 0.746 | 1.762 | 0.041 | 0.047 |
| RM223 | 16 | 0.516 | 0.079 | 1.071 | 0.848 | 0.103 |
| RM333 | 30 | 0.727 | 0.612 | 1.763 | 0.089 | 0.094 |
| Mean | 15.9 | 0.629 | 0.254 | 1.325 | 0.620 | 0.114 |
| Std. Error | 0.891 | 0.032 | 0.043 | 0.099 | 0.061 | 0.014 |

He=expected heterozygosity, Ho=observed heterozygosity, I=Shannon's information index, F=fixation index, Fst=fixation index statistic.

determine diversity in the rice genotypes (Table 3). All 10 markers were polymorphic, detecting a total of 159 alleles ranging from 6 to 30 alleles per locus with an average of 15.9±0.9 alleles/locus, clearly indicating that this set of 10 markers revealed a high level of genetic variability throughout the germplasm. The markers were picked from different linkage groups (chromosomes) of the rice genome and from the results, they amplified a varying number of alleles. The number of alleles observed in this research for markers RM125 (7) and RM7 (6) are comparable to those noted by earlier researchers on African rice (Drame et al., 2011). However, there were more alleles revealed in this study for markers RM333 (30 alleles), RM154 (21 alleles) and RM249 (19 alleles), indicating greater diversity in the materials used. This could be due to the use of breeding materials generated by intercrossing IRRI, AfricaRice and CIAT lines, thus increasing their level of diversity or due to materials having different origins. Other studies that considered different origins include those of Semon et al. (2005) who worked on 198 *Oryza glaberrima* accessions from 12 countries using 93 SSR markers, and reported an average of 9.4 alleles, Drame et al. (2011) studied 74 *O. glaberrima* samples from 9 countries used 30 SSR markers, and reported an average of 8.4 alleles. These differing results can probably be explained by differences in the sampling and number of markers used in the study. The most informative markers were RM 333, RM 20A and RM 206 since they were able to identify rare alleles and therefore give the highest numbers at 30, 23 and 24

alleles, respectively.

For all loci, the observed Heterozygosity (Ho) was lower (mean = 0.254 ± 0.043) than the expected Heterozygosity (He) (mean = 0.629 ± 0.032), suggesting a clear shift from the Hardy-Weinberg equilibrium. This shift can only be attributed to forces akin to inbreeding within groups (Masudaab et al., 2009) or lack of distinctly isolated populations of the available rice germplasm in Uganda. The Fst values were generally low, ranging from 0.047 to 0.192, indicating that low levels of genetic differentiation were present in the populations sampled from IRRI, AfricaRice and NaCRRI, as reported by Ogumbayo et al. (2005) regarding genotypes from AfricaRice.

## Diversity groups of Uganda rice genotypes

Grouping of the rice populations was determined using ancestry-based grouping and a phylogenetic tree identifying the different groups. Using a cluster analysis of the allelic data (Figure 1), five genetic groups were identified in the rice germplasm used in the study. The classification of the genotypes was in agreement with their parentage, which comprised of *Aus*, *Indica*, tropical *japonica*, temperate *Japonica* and *Basmati* groups of rice. A similar population structure was also documented by Garris and Ni (Garris et al., 2005; Ni et al., 2002). Garris et al. (2005) reported 5 populations that corresponded to *indica*, *aus*, *aromatic*, *temperate japonica* and *tropical japonica* using samples from Asia, the Americas and Africa. The statistical program used in this study (STRUCTURE V2.3.4) tends to give more populations that are biologically relevant. Therefore, to ascertain the identified groups, the method described by Evanno et al. (2005) was used.

This method tests the number of populations that are statistically significant in the samples when patterns of dispersal among them are not homogeneous as in the case of allelic variability (Evanno et al., 2005). By using the log probability for the rate of change of the data (ΔK) between values of successive K (number of populations), STRUCTURE gave the accurate number of populations (K) at K=5. The results, presented in Figure 2, revealed the structure of the rice genotypes showing five populations consisting of admixed genotypes. This admixture in populations is probably because rice breeding centers share germplasm freely and make crosses with whichever cultivars show desirable traits, resulting in common alleles in all populations (Ni et al., 2002). This gene flow (gene migration) causes a marked change in allelic frequency (Beringer, 2003), so that alleles of various individuals end up being present in all populations. There was therefore no “island” population of rice genotypes observed in this study, indicating that the genetic base was small, as suggested by Cuevas-Pe´rez et al. (1992).

The genotypes grouped according to differences in their alleles, revealed one extra group compared to Drame et al. (2011) who reported four distinct genetic groups of African rice samples using samples of wild rice (*O. glaberrima*, *Oryza longistaminata* and *Oryza barthi*) and 6 cultivated genotypes. However, the present study used cultivated rice, some of which were NERICA varieties that are crosses between wild and cultivated rice, and therefore were expected to have broader genetic diversity than shown in the earlier studies by Drame et al. (2011). The groups observed in this study were not clearly separated from each other, due to admixtures, which suggested that most of them were crosses with each other. Genotypes 11 (IR 83372-B-B-133-2), 3 (BGM) and 57 (NERICA 1/WAC 117) clustered alone in group 1 (Figure 1) suggesting a unique group of alleles that was not present in other groups, and thus indicating that this group could be useful as parents to broaden the diversity of rice breeding materials (Cuevas-Pe´rez et al., 1992).

## Assigning genotypes to their centers of origin

The AMOVA results are presented in Table 4, revealing most of the variation among individual genotypes (56%) and within individuals (41%). Only 3% of the variation was associated with the origins of the genotypes, suggesting that the diversity available among and within the rice genotypes was sufficient to be used for improving rice productivity in Uganda. The four origins of the 99 rice genotypes used in this study were; NaCRRI (61), IRRI (24), AfricaRice (12) and Ugandan farmers (2) and analysis using GenAlEx software was expected to assign the genotypes to their respective centers of origin. The results (Table 5) indicated that the different centers of origin of the genotypes, as their fixed population in the analysis, were assigned 53% while 47% of them were assigned to other populations (Other) at $P < 0.1$. The markers used were able to differentiate half of the genotypes used by their centers of origin. The other 47% were assigned different origins probably because they resulted from crosses between genotypes that could have been introduced from other centers (Guimaraes, 2009). The origin of neither of the two varieties obtained from farmers in Uganda (Supa and Sindano) was assigned to farmers, and remains unclear (Kijima et al., 2012) though they are believed to have originated from Tanzania (Kijima et al., 2012) where Sindano is a landrace (FAO, 1987).

## Conclusion

The use of molecular markers to determine genetic diversity in the germplasm is demonstrated as a feasible approach in Uganda. The SSR markers RM 333, RM 20A and RM 206 were the most informative in this research.

**Figure 1.** UWNJ tree cluster analysis using DARwin V5 software.

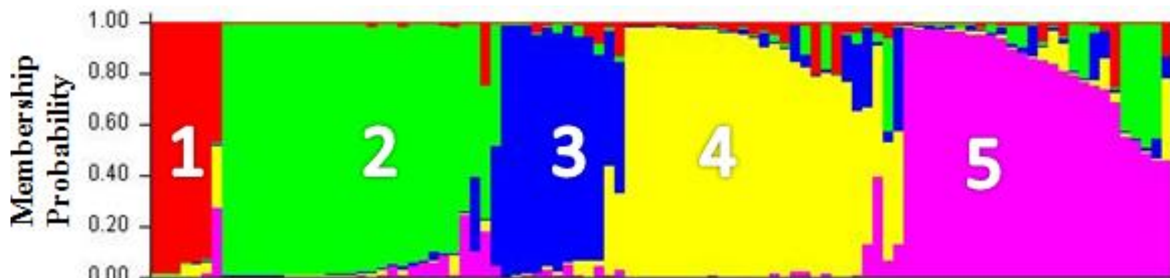


**Figure 2.** Population Structure of 99 rice genotypes at k=5.

**Table 4.** Summary of AMOVA in the genotypes.

| Source | df | SS | MS | Est. Var. | %Var. |
|---|---|---|---|---|---|
| Among Origins | 3 | 28.633 | 9.544 | 0.102 | 3% |
| Among Individuals | 95 | 555.716 | 5.850 | 2.147 | 56% |
| Within Individuals | 99 | 154.000 | 1.556 | 1.556 | 41% |
| Total | 197 | 738.348 | | 3.805 | 100% |

A probability value of 0.001 was used based on permutation across the full data set.

**Table 5.** Assignment of population to origin or "other" categories.

| Population | Genotypes in Population | Origin Population assignment | Other Population assignment | Probability level |
|---|---|---|---|---|
| NaCRRI | 61 | 41 | 20 | |
| IRRI | 24 | 8 | 16 | |
| AfricaRice | 12 | 3 | 9 | |
| Uganda-Farmers | 2 | 0 | 2 | |
| **Total** | **99** | **52** | **47** | |
| Percent | 100% | 53% | 47% | 99% |

The rice germplasm from IRRI, AfricaRice and NaCRRI was assigned 5 groups.

## Conflict of interests

The authors declare that there are no conflicts of interests

## ACKNOWLEDGEMENTS

We are grateful to the administration of the University of Pretoria for allowing us to use their facilities at the Forestry and Agricultural Biotechnology Institute (FABI). We sincerely thank Mrs Renete Zipfel and Miss Gladys Shobangu for training the first author in the use of GeneMapper V4.1 software and hands-on assistance in the genotyping work. This work was financially supported by the World Bank through the Eastern Africa Agricultural Productivity Project (EAAPP) of the National Agricultural Research Organization (NARO), Uganda.

**Abbreviations: IRRI,** International Rice Research Institute; **SSR,** simple sequence repeat; **NaCRRI,** national crops resources research institute; **RAPD,** random amplified polymorphic DNA; **AFLP,** amplified fragment length polymorphisms; **RFLP,** restriction fragment length polymorphisms; **SNP,** single nucleotide polymorphisms; **BAC,** bacterial artificial chromosome; **PAC,** P1-derived artificial chromosome; **PCR,** polymerase chain reaction; **Ho,** heterozygosity; **He,** heterozygosity.

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academicJournals

Vol. 14(18), pp. 1543-1551, 6 May, 2015
DOI: 10.5897/AJB2015.14567
Article Number: FC7397852709
ISSN 1684-5315
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http://www.academicjournals.org/AJB

African Journal of Biotechnology

*Full Length Research Paper*

# Molecular diversity study of black cumin (*Nigella sativa* L.) from Ethiopia as revealed by inter simple sequence repeat (ISSR) markers

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Received 14 March, 2015; Accepted 27 April, 2015

***Nigella sativa* L. (commonly known as black cumin) belonging to family Rannunculaceae is an important medicinal plant with worldwide distribution. In Ethiopia, *N. sativa* occurs in all regions and agro-ecologies at different altitudinal ranges. This plant has a lot of importance in Ethiopia. However, there is no information available on molecular genetic diversity of this crop in respect to Ethiopia. Therefore, the aim of this study was to investigate the genetic diversity of black cumin populations collected from Amhara, Oromia, Tigray, Benshangul and South Nation's Nationality People regions of Ethiopia using ISSR marker. A total of 84 black cumin accessions were obtained from Institute of Biodiversity Conservation (IBC). The ISSR marker was used for computing gene diversity, percent polymorphism, Shannon diversity index and AMOVA. Overall, accessions from Oromia showed the highest gene diversity (H = 0.35) and Shannon information index (I= 0.52), followed by Amhara with gene diversity and Shannon index values of (0.35) and (0.51), respectively. NJ and UPGMA results showed strong grouping among accessions collected from the Oromia and Amhara region. The five geographical regions of Ethiopia showed different levels of genetic variation. Thus, conservation priority should be given for those regions that have low genetic diversity.**

**Key words:** Conservation, indigenous, molecular markers, primers.

## INTRODUCTION

The genus Nigella contains about 14 species of annual herbs. Among the medicinal plants in use from prehistoric times, black cumin (*Nigella sativa*) is being used for healthcare. It is commonly known as black cumin, fennel

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flower, Roman coriander and Kalonji (Weiss, 2002). Different bioactive compounds have been reported in this spice and these are well known for their beneficial effects on health (Hussain et al., 2006). These contain essential macro and micronutrients which play vital roles as structural and functional components of metalloproteins and enzymes in the living cells (Ansari et al., 2004).

*N. sativa* has been used traditionally for centuries in the Middle East, Northern and east Africa and India for the treatment of various diseases (Worthen, 1998; Burits and Bucar, 2000; Al-Ghamdi, 2001; Gilani et al., 2004). Research from around the globe is giving increasing support for black cumins widespread healing powers. Extracts of the black cumin have many therapeutic effects such as anti-diabetes, antibacterial, and antitumor (Khan et al., 2003; Kanter et al., 2003; Hussein et al., 2005).

Assessment of the genetic diversity in black cumin is therefore, of crucial importance for developing conservation strategy for this economically important spice species. With the development of the polymerase chain reaction (PCR), many molecular techniques have been, and still are being developed for plant genome analysis (Esayas and Bryngelsson, 2006). They have been replacing the traditional morphological and agronomic characterization, since they virtually cover the whole genome, are not influenced by the environment, and less time consuming (Esayas et al., 2005).

Molecular techniques have been applied in the analysis of specific genes, as well as to increase understanding of gene action, generate genetic maps and assist in the development of gene transfer technologies. Molecular techniques have also had critical roles in studies of phylogeny and species evolution, and have been applied to increase our understanding of the distribution and extent of genetic variation within and between species. Markers such as inter-simple sequence repeat (ISSR) (Zietkiewicz et al., 1994) are widely used in genetic diversity studies because they need no prior DNA sequence information, development costs are low and laboratory procedures can easily be transferred to any plant species.

Black cumin is found in different parts of Ethiopia at various altitude ranges. This shows that there is the existence of genetic diversity of black cumin species that deserve conservation. However, no information on molecular genetic diversity of this herb has been generated for the Ethiopian population. One of the important factors restricting their large-scale production and development of better varieties is that very little information is available about their genetic diversity, inter and intra-specific variability and genetic relationships among these species. Therefore, attempts to analyze possible untapped genetic diversity become extremely essential for breeding and crop improvement. This study investigated the genetic diversity and population structure of black cumin germplasms collected from Ethiopia using ISSR markers.

## MATERIALS AND METHODS

### Sampling locations and plant materials

The present study was conducted at Genetics, laboratory Department of Microbial, Cellular and Molecular Biology, Addis Ababa University. A total of 84 *N. sativa* accessions were provided by Institute of Biodiversity Conservation (IBC). The samples were originally collected from different parts of Ethiopia. Soil was prepared with a PH range from 5-8 and all accessions were planted in pots at College of Natural Sciences greenhouse and watering every day to maintain the normal growth of the plant in the green house for fifteen days. A total of 34, 24, 10, 9 and 7 accessions were included from Oromia, Amhara, Benshangul gumuz, Tigray and South Nation's Nationality Peoples Regions, respectively (Table 1).

### DNA extraction

The study was designed to characterize the 84 black cumin accessions using inter simple sequence repeat (ISSR) markers. Young fresh leaves were collected separately from seven randomly selected individual plants per accession after three weeks of planting in the greenhouse. Total genomic DNA was isolated from about 0.4 g of the pulverized leaf sample using modified triple Cetyl Trimethyl Ammonium Bromide (CTAB) extraction technique as described by Borsch et al. (2003).

### ISSR Primer selection and optimization

A total of ten primers, obtained from the Genetics Laboratory (Primer kit UBC 900) were initially tested for primers variability and reproducibility through optimization process. The optimization process was repeated three times to found the optimum conditions of the PCR and the same band were observed. To represent wider geographic locations, two individuals from Amhara, two from Oromia, two from Benshangul Gumuz, two from SNNP and two from Tigray were selected to screen the ten primers. Finally, a total of five polymorphic and reproducible ISSR primers (UBC-809, UBC-810, UBC-811, UBC-835 and UBC- 880) were selected after testing its reproducibility and polymorphism.

### PCR and gel electrophoresis

The polymerase chain reaction was conducted in Biometra 2003 T3 Thermo cycler. PCR amplification was carried out in a 25 µl reaction mixture containing 1 µl template DNA, 17.8 µl $H_20$, 0.2 µl dNTP (20 mM), 2.6 µl Taq buffer (10X buffer B), 2.5 µl $MgCl_2$ (25 mM), 0.6 µl primer (20 pmol/ l) and 0.3 µl Taq Polymerase (5 u/µl) for all the five primer namely 809, 810, 811, 835 and 880. The amplification program was 4 min preheating, initial denaturation at 94°C, then 40 x 15 s at 94°C, 1 min primer annealing at (45°C/ 48°C) based on primers used, 1.30 min extension at 72°C and the final extension for 7 min at 72°C. The PCR reactions were then stored at 4°C until loading on gel for electrophoresis.

The amplification products were separated by electrophoresis using an agarose gel (1.67% agarose with 100 ml 1xTBE) with 8 µl amplification product of each sample plus 2 µl loading dye (6X concentrated) was loaded on gel. DNA marker with 3000 bp was used to estimate molecular weight and size of the fragments. Electrophoreses was done for 3 h at constant voltage of 100 V. The ISSR gel was then stained with 10 mg/ml ethidum bromide which

**Table 1.** *N. sativa* accessions, latitude, longitude and regions of collection used in the study.

| Number | Accession | Region | Zone | Latitude | Longitude | Symbol | |
|---|---|---|---|---|---|---|---|
| 1 | 8502 | Oromia | Bale | 37-00-00-N | 39-48-00-E | T1 | oro-1 |
| 2 | 9067 | Amhara | Mirab Gojam | 11-41-08-N | 37-01-12-E | T2 | amh-1 |
| 3 | 9068 | Amhara | Mirab Gojam | 11-45-40-N | 37-05-4 –E | T3 | amh-2 |
| 4 | 9069 | Amhara | Mirab Gojam | 10-38-48-N | 37-05-09-E | T4 | amh-3 |
| 5 | 9071 | Amhara | Mirab Gojam | 10-38-21-N | 37-05-13-E | T5 | amh-4 |
| 6 | 90501 | Amhara | Mirab Gojam | 10-38-21-N | 37-05-13-E | T6 | amh-5 |
| 7 | 90502 | Amhara | Debub Gondar | 11-57-00-N | 37-42-00-E | T7 | amh-6 |
| 8 | 90503 | Amhara | Debub Gondar | 11-59-00-N | 37-46-00-E | T8 | amh-7 |
| 9 | 90504 | Oromia | Arssi | 08-03-N | 38-47-00 | T9 | oro-2 |
| 10 | 90505 | Amhara | Misrak Gojam | 10-20-00-N | 38-00-00-E | T10 | amh-8 |
| 11 | 90506 | Amhara | Misrak Gojam | 10-20-00-N | 38-00-00-E | T11 | amh-9 |
| 12 | 90507 | Amhara | Semen Gondar | 12-16-00-N | 37-05-00-E | T12 | amh-10 |
| 13 | 90508 | Oromia | Mirab Wellega | 09-00-00-N | 35-15-00-E | T13 | oro-3 |
| 14 | 90509 | Oromia | Bale | 07-19-00-N | 39-48-00-E | T14 | oro-4 |
| 15 | 90510 | Oromia | Mirab Shewa | 09-10-00-N | 37-50-00-E | T15 | oro-5 |
| 16 | 90511 | Oromiya | Arssi | 08-03-N | 38-47-00 | T16 | oro-6 |
| 17 | 90512 | Benshangul Gumuz | Metekel | 08-22-00-N | 39-53-00-E | T17 | bgu-1 |
| 18 | 90513 | Oromia | Arsi | 08-01-00-N | 39-50-00-E | T18 | oro-7 |
| 19 | 90514 | Benishangul Gumuz | Metekel | 11-00-00-N | 35-45-45-E | T19 | bgu-2 |
| 20 | 90515 | Tigray | Misrakawi | 13-03-00-N | 39-13-00-E | T20 | tig-1 |
| 21 | 90516 | Oromia | Bale | 07-01-00-N | 39-59-00-E | T21 | Oro-8 |
| 22 | 90517 | SNNP | Semen Omo | 60-60-54-N | 36-87-94-E | T22 | snn-1 |
| 23 | 205167 | SNNP | Semen Omo | 60-60-54-N | 36-87-94-E | T23 | snn-2 |
| 24 | 207538 | Amhara | Semen Gondar | 12-20-00-N | 37-14-00-E | T24 | amh-11 |
| 25 | 207539 | Amhara | Semen Gondar | 12-20-00-N | 37-14-00-E | T25 | amh-12 |
| 26 | 207540 | Amhara | Debub Gondar | 12-20-00-N | 37-14-00-E | T26 | amh-13 |
| 27 | 208032 | Amhara | Semen Gondar | 12-20-00-N | 37-14-00-E | T27 | amh-14 |
| 28 | 208688 | Oromia | Mirab Harerge | 08-49-00-N | 40-25-00-E | T28 | oro-9 |
| 29 | 208771 | Oromia | Mirab Wellega | 37-56-25-N | 38-67-11-E | T29 | oro-10 |
| 30 | 212520 | Tigray | Egnaw | 14-06-00-N | 38-42-00-E | T30 | tig-2 |
| 31 | 212859 | Oromia | Bale | 07-01-00-N | 39-59-00-E | T31 | oro-11 |
| 32 | 212437 | Benshangul Gumuz | Asosa | 10-03-44-N | 34-32-50-E | T32 | bgu-3 |
| 33 | 215319 | Amhara | Misrak Gojam | 11-00-08-N | 37-00-11-E | T33 | amh-15 |
| 34 | 219970 | Tigray | Mirabawi | 14-08-12-N | 38-18-34-E | T34 | tig-3 |
| 35 | 223069 | Amhara | Misrak Gojam | 11-00-08-N | 37-00-11-E | T35 | amh-16 |
| 36 | 223070 | Benishangul Gumuz | Metekel | 11-00-00-N | 35-45-45-E | T36 | bgu-4 |
| 37 | 223071 | Benishangul Gumuz | Metekel | 11-00-00-N | 35-45-45-E | T37 | bgu-5 |
| 38 | 223072 | Benishangul Gumuz | Metekel | 11-00-00-N | 35-45-45-E | T38 | bgu-6 |
| 39 | 229806 | Benishangul Gumuz | Asosa | 10-03-44-N | 34-32-50-E | T38 | bgu-7 |
| 40 | 229807 | Benishangul Gumuz | Asosa | 10-04-48-N | 36-31-40-E | T40 | bgu-8 |
| 41 | 230037 | Tigray | Egnaw | 14-06-00-N | 38-42-00-E | T41 | tig-4 |
| 42 | 230038 | Tigray | Egnaw | 14-10-00-N | 38-45-00-E | T42 | tig-5 |
| 43 | 230039 | Tigray | Egnaw | 14-04-00-N | 38-05-00-E | T43 | tig-6 |
| 44 | 230040 | Tigray | Egnaw | 14-05-00-N | 39-06-00-E | T44 | tig-7 |
| 45 | 230777 | Oromia | Borena | 05-07-00-N | 39-29-00-E | T45 | oro-12 |
| 46 | 236832 | Oromia | Mirab Shewa | 38-01-00-N | 38-05-00-E | T46 | oro-13 |
| 47 | 237989 | Oromia | Bale | 08-03-N | 38-47-00 | T47 | oro-14 |
| 48 | 239730 | Oromia | Bale | 07-00-03-N | 40-27-68-E | T48 | oro-15 |
| 49 | 240403 | SNNP | Keficho Shekicho | 07-13-63-N | 35-41-93-E | T49 | snn-3 |
| 50 | 240404 | SNNP | Keficho Shekicho | 07-15-00-N | 36-00-00-E | T50 | snn-4 |
| 51 | 242220 | Amhara | Debub Wello | 10-50-28-N | 39-48-60-E | T51 | amh-17 |

**Table 1.** Contd

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 52 | 242221 | Amhara | Debub Wello | 10-50-28-N | 39-48-60-E | T52 | amh-18 |
| 53 | 242222 | Amhara | Semen Wello | 11-57-65-N | 39-03-80-E | T53 | amh-19 |
| 54 | 242223 | Tigray | Mirabawi | 14-06-75-N | 38-27-89-E | T54 | tig-8 |
| 55 | 242224 | SNNP | Arbaminch | 06-06-67-N | 37-66-67-E | T55 | snn-5 |
| 56 | 242225 | SNNP | Arbaminch | 06-06-67-N | 37-66-67-E | T56 | snn-6 |
| 57 | 242226 | SNNP | Soddo | 06-51-10-N | 37-45-40-E | T57 | snn-7 |
| 58 | 242227 | SNNP | Soddo | 10-54-32-N | 39-47-29-E | T58 | snn-8 |
| 59 | 242528 | Benishangul Gumuz | Asosa | 09-59-09-N | 34-40-03-E | T59 | bgu-9 |
| 60 | 242824 | Tigray | Debubawi | 12-13-00-N | 38-43-00-E | T60 | tig-9 |
| 61 | 242825 | Oromia | Arssi | 07-35-00-N | 39-32-00-E | T61 | Oro-16 |
| 62 | 242826 | Oromia | Arssi | 07-40-00-N | 40-12-00-E | T62 | Oro-17 |
| 63 | 242827 | Oromia | Bale | 07-17-00-N | 39-50-00-E | T63 | Oro-18 |
| 64 | 242828 | Oromia | Bale | 06-58-00-N | 40-33-00-E | T64 | Oro-19 |
| 65 | 242829 | Oromia | Bale | 07-07-00-N | 40-36-00-E | T65 | Oro-20 |
| 66 | 242830 | Oromia | Bale | 06-53-00-N | 40-42-00-E | T66 | Oro-21 |
| 67 | 242831 | Oromia | Bale | 06-56-00-N | 40-39-00-E | T67 | Oro-22 |
| 68 | 242832 | Oromia | Borena | 04-58-00-N | 38-13-00-E | T68 | Oro-23 |
| 69 | 242833 | Oromia | Arssi | 07-39-03-N | 39-29-46-E | T69 | Oro-24 |
| 70 | 242834 | Oromia | Arssi | 07-35-73-N | 39-33-43-E | T70 | Oro-25 |
| 71 | 242835 | Oromia | Arssi | 07-35-73-N | 39-33-49-E | T71 | Oro-26 |
| 72 | 242836 | Oromia | Arssi | 07-36-22-N | 39-33-04-E | T72 | Oro-27 |
| 73 | 242837 | Oromia | Arssi | 07-36-22-N | 39-33-04-E | T73 | Oro-28 |
| 74 | 242838 | Oromia | Arssi | 07-35-71-N | 39-32-29-E | T74 | Oro-29 |
| 75 | 242839 | Oromia | Arssi | 07-33-79-N | 39-31-42-E | T75 | Oro-30 |
| 76 | 242840 | Oromia | Arssi | 07-33-16-N | 39-31-60-E | T76 | Oro-31 |
| 77 | 242841 | Oromia | Arssi | 07-32-64-N | 39-31-87-E | T77 | Oro-32 |
| 78 | 242842 | Oromia | Arssi | 07-32-08-N | 39-32-11-E | T78 | Oro-33 |
| 79 | 242843 | Oromia | Arssi | 07-32-08-N | 39-32-11-E | T79 | Oro-34 |
| 80 | 242844 | Benishangul Gumuz | Asosa | 07-36-22-N | 39-31-24-E | T80 | bgu-10 |
| 81 | 242845 | Amhara | Semen Gonder | 07-35-53-N | 39-30-73-E | T81 | Amh-21 |
| 82 | 242846 | Amhara | Semen Gonder | 07-35-87-N | 39-29-33-E | T82 | Amh-22 |
| 83 | 244653 | Amhara | Semen Gondar | 12-50-00-N | 37-35-00-E | T83 | Amh-23 |
| 84 | 244654 | Amhara | Semen Gondar | 12-50-00-N | 37-05-00-E | T84 | Amh-24 |

was mixed with 250 ml distilled water for 30 min and distained with distilled water for 30 min.

**Data analysis**

ISSR fragments were scored visually for each individual accession from the gel photograph. The fragments were scored as discrete characters; presence 1 or absence 0 data. Based on recorded bands, different software's are used for statistical analysis. POPGENE version1.32 software was used to calculate genetic diversity for each population (accessions were grouped based on their geographic origin), number of polymorphic loci, percent polymorphism, gene diversity (H) and Shannon diversity index (I) (Yeh et al., 1999).

Analysis of molecular variance (AMOVA) was also used to calculate variation among and within population using Areliquin version 3.01 (Excoffier et al., 2006). The unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973) was used to analyze and compare the population and generate phenogram using NTSYS- pc version 2.02 (Rohlf, 2000). The Neighbor Joining (NJ) method (Saitou and Nei, 1987; Studier and Keppler, 1988) was used to compare individual genotypes and evaluate patterns of genotype clustering using Free Tree 0.9.1.50 Software (Pavlicek et al., 1999).

## RESULTS AND DISCUSSION

### ISSR Primers and their banding patterns

Out of the ten ISSR primers initially tested, only five primers (UBC-809, UBC-810, UBC-811 and UBC-835 and UBC-880) that produced clear, reproducible and polymorphic bands were selected as informative primers (Table 3). The other five ISSR primers that resulted in either smeared, failed to give amplification products, poor or non-reproducible bands were excluded from the

**Table 2.** Primers, annealing temperature, primer sequence, amplification quality and repeat motives used for optimization.

| Primer | Annealing temperature (°C) | Primer sequence | Amplification quality | Repeat motives | Status of Primers |
|---|---|---|---|---|---|
| 809 | 48 | (AG)8G | Polymorphic reproducible | Dinucleotide | selected |
| 810 | 45 | (GA)8T | Polymorphic reproducible | Dinucleotide | selected |
| 811 | 48 | (GA)8C | Polymorphic reproducible | Dinucleotide | selected |
| 812 | 45 | (GA)8A | Monomorphic, reproducible | Dinucleotide | Not selected |
| 818 | 48 | (CA)8G | Monomorphic, reproducible | Dinucleotide | Not selected |
| 826 | 48 | (AC)8C | Polymorphic, reproducible | Dinucleotide | Selected |
| 834 | 45 | (AG)8YT | Polymorphic, reproducible | Dinucleotide | Selected |
| 835 | 48 | (AG)8YC | Polymorphic, reproducible | Dinucleotide | Selected |
| 873 | 45 | (GACA)4 | monomorphic, reproducible | Tetra- nucleotide | Not Selected |
| 880 | 45 | (GGAGA)3 | Polymorphic, reproducible | Penta- nucleotide | Selected |

**Table 3.** Banding patterns generated using the five selected primers, amplification quality and number of scored bands.

| Primers | Primer Sequence | Amplification Quality | Number of scored bands |
|---|---|---|---|
| UBC-809 | (AG)8G | Good | 13 |
| UBC-810 | (GA)8T | Very good | 18 |
| UBC-811 | (GA)8C | Good | 12 |
| UBC-835 | (AG)8YC | Very good | 17 |
| UBC-880 | (GGAGA)3 | Good | 12 |
| Total | | | 72 |

Single-letter abbreviations for mixed base positions: R = (A, G) Y = (C, T)

analysis. The amplicon (band) size of the five primers used in this study varied from 200 to 1400 bp (Table 2).

A total of 72 clear and scorable bands were recorded for the 84 black cumin accessions of which 72 (100%) were found to be polymorphic. The least number of scorable bands were scored for primer UBC-811 and UBC-880 (12); the highest number of bands were scored from primer UBC-810 (18), whereas 17 and 13 numbers of bands were scored for primer UBC-835 and UBC-809, respectively. The average number of bands and polymorphic bands per primer was 14.4 (Table 3).

The Nei's gene diversity (H) and the Shannon Information Index (I) calculated for each of the ISSR Primers showed Primer UBC-835 had the lowest gene diversity (0.31); primer UBC-809 had the highest gene diversity (0.43). UBC-880, UBC-811 and UBC- 810 had (0.42), (0.37) and (0.33) Nei's gene diversity. The Shannon's Information index ranged from 0.46 to 0.62. In the case of genetic diversity estimation which was due to gene diversity (h) and Shannon's information index (I), the highest values were from ISSR-880 primer and the least were ISSR-835 (Table 4).

The black cumin populations with the highest values of Nei's and the Shannon index were Amhara populations with 0.35 and 0.51, respectively and SNNP populations were the lowest (0.25 and 0.37, respectively for gene diversity and Shannon's Information index). The highest level of percent polymorphism was obtained from samples collected from Oromia (100) followed by Amhara, Tigray, Benshangul Gumuz and SNNPR; 88.89, 75, 70.83 and 63.89, respectively (Table 4).

**Table 4.** Number of scorable bands (NSB), number of polymorphic loci (NPL), percent polymorphism (PPL), gene diversity (H), Shanon information Index (I) of black cumin accessions in the present study.

| Primers | NSB | NPL | PPL | H±SD | I±SD |
|---|---|---|---|---|---|
| UBC-809 | 13 | 13 | 100 | 0.43±0.10 | 0.62±0.12 |
| UBC-810 | 18 | 18 | 100 | 0.32±0.16 | 0.48±0.20 |
| UBC-811 | 12 | 12 | 100 | 0.37±0.16 | 0.55±0.21 |
| UBC-835 | 17 | 17 | 100 | 0.31±0.14 | 0.47±0.17 |
| UBC-880 | 12 | 12 | 100 | 0.42±0.11 | 0.61±0.13 |
| Over all | 72 | 72 | 100 | 0.42±0.11 | 0.54±0.17 |

In regions based analysis, accessions collected from Oromia showed the highest number of polymorphism with 100% polymorphism followed by Amhara with 88.89% polymorphism followed by Tigray with 75% polymorphism

**Table 5.** The number of polymorphic loci (NPL), percent polymorphism (PP), and genetic diversity (H) and Shannon information index (I) of black cumin accessions in the five regions.

| Regions | Number of samples (N) | NPL | PP | H±SD | I±SD |
|---|---|---|---|---|---|
| Oromia | 34 | 72 | 100.00 | 0.36±0.13 | 0.52±0.16 |
| Amhara | 24 | 64 | 88.89 | 0.35±0.18 | 0.51±0.24 |
| Benshangul | 10 | 51 | 70.83 | 0.26±0.21 | 0.38±0.28 |
| Tigray | 9 | 54 | 75.00 | 0.26±0.19 | 0.39±0.27 |
| SNNP | 7 | 46 | 63.89 | 0.25±0.21 | 0.36±0.29 |
| Overall | 84 | 57.4 | 79.70 | 0.29±0.18 | 0.44±0.25 |

**Table 6.** Analysis of Molecular Variance (AMOVA) of black cumin accessions in Ethiopia without grouping into regions of origin.

| Variation source | Sum of squares | Variation Components | Percentage of variation | P |
|---|---|---|---|---|
| Among Population | 97.34 | 0.72 | 4.96 | 0.001 |
| Within Population | 1059.24 | 13.43 | 95.04 | |
| Total | 1156.58 | 14.15 | 100 | |

followed by Benshangul Gumuz with 70.83%. The least percent polymorphism was detected in the accessions collected from SNNP region with 63.89% polymorphism (Table 5).

Similarly, black cumin samples from Amhara exhibited the highest gene diversity (H= 0.35) followed by accession from Oromia, Benshangul Gumuz, Tigray and SNNP with 0.35, 0.26, 0.26 and 0.25 values, respectively. In general, Oromia and Amhara had good genetic diversity than Benshangul Gumuz,Tigray and SNNPR. But this has to be further studied using proper sampling strategy and multi-location comparison (Table 5).

## Analysis of molecular variance

Analysis of molecular variance was carried out on the overall ISSR data score of black cumin accessions without grouping by region or geographic location (Table 6). Accordingly, AMOVA revealed that 95.04269% of the variation was within population while 4.95731% of variation remained among population. The variation is highly significant at P = 0.001. Unlike other landraces of cultivated plants, *N. sativa* in Ethiopia is not restricted to a given area rather it is wildly exchanged among local community and markets. This could be caused by relatively higher seed exchange rate among different regions at its regional and central markets which could lead to the intermix of genotypes (Polhil and Raven, 1981; Davoud et al., 2010) and also defined insect pollinators could be another agent to facilitate gene flow among populations.

Genetic diversity of plant populations is largely influenced by factors such as reproduction system, genetic drift, evolutionary history and life history (Loveless and Hamrick, 1984). In broad-spectrum, out crossing species have higher levels of genetic diversity than selfing and clonal plants (Rossetto et al., 1995).

The present study also confirmed the presence of higher diversity as revealed by higher percent of polymorphism which is higher than Tunisian fenugreek germplasms (Nidhal et al., 2011) (94.12%). The present study also revealed higher polymorphism than said by Rakhee et al. (2004) in which 72% of polymorphism for *T. foenum graecum* was revealed by ISSR markers. Mohammed and Tesfaye (2015) studied the spice plant on *Lepidium sativum* populations by using ISSR marker and showed that there was 94% within population and very low genetic diversity among population (6%). The present study showed higher percent polymorphism and higher proportion of diversity within population of *N. sativa* comparable with that ascertained by Mohammed and Tesfaye (2015). Tewodros (2013) studied other spice plant fenugreek (*T. foenum-graecum*) populations by using ISSR marker and showed 64.1% polymorphism within populations and 35.9% polymorphism among populations. As compared to the present study, there was difference between the two spices according to AMOVA analysis. Thus, in the present study there was high gene flow among regions by different mechanisms.

## Clustering analysis

UPGMA analysis based on regions of collection of *N. Sativa* revealed two major groups. The first cluster

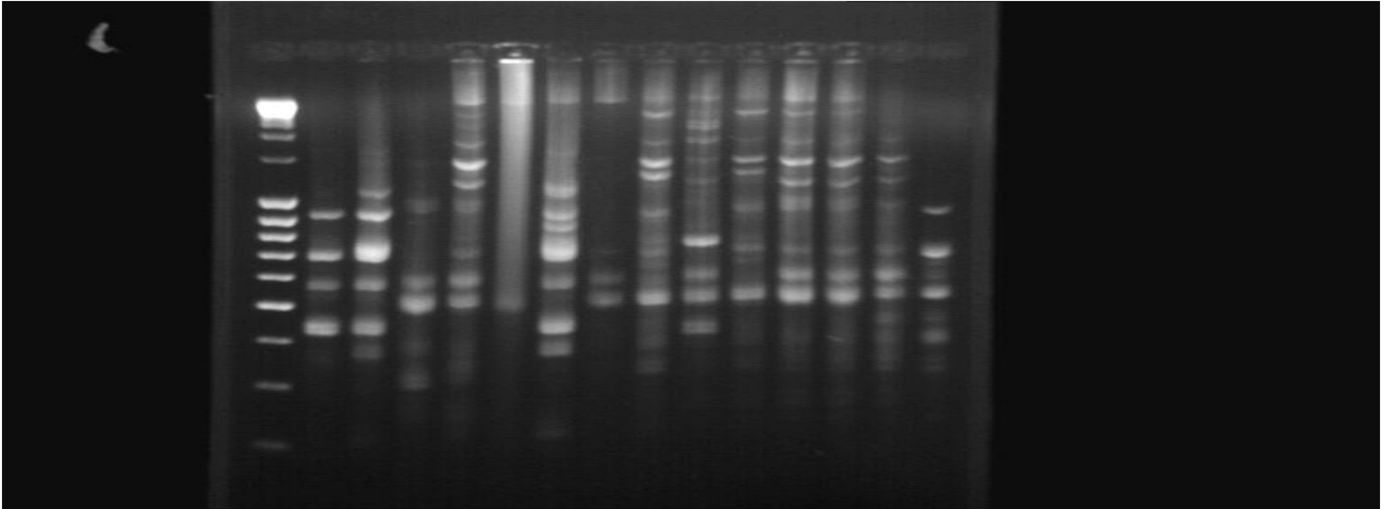

**Figure 1.** ISSR fingerprint generated from 14 individual accessions using primer UBC- 809.

**Figure 2.** UPGMA based dendrogram for 5 *N. sativa* populations using 5 ISSR (4 di, 1 penta nucleotide) primers.

**Figure 3.** Neighbor-joining result of 84 individuals based on 72 PCR bands amplified by 4 di nucleotide (809, 810,811, and 835), and 1 penta nucleotide (880) primers using Jaccard's coefficient. Oro = Oromia; Amh = Amhara; Bgu=benshangul Gumuz; Tig = Tigray; snn=south nation's nationality peoples.

contains Oromia and Amhara while the second cluster contains Benshangul Gumuz, Tigray and SNNP. UPGMA with individual accessions showed group formation or clustering based on regions (Figure 2).

All individual accessions collected from Oromia and accessions collected from Amhara region tend to form their own groups. On the other hand, those accessions collected from Benshangul gumuz, Tigray and SNNP region mixed with each other's (Figure 3).

## Conclusion

*N. sativa* is cultivated in different world parts including

Ethiopia. *N. sativa* is have medicinal value among local people. Because of insufficient amount of modern medicine, most of the Ethiopia population relies on traditional medicine. In Ethiopia, it is not commonly cultivated using separate farm plot rather planted along with different boarder crop. The present study was conducted with the main objective of assessing the extent of genetic diversity among Ethiopian accessions of *N. sativa* provided by the Institute of Biodiversity Conservation (IBC) using Inter Simple Sequence Repeat marker. This study shows that, ISSR markers are important markers for genetic diversity study in *N. sativa* accessions. Accessions collected from Oromia and Amhara regions were highly diversified than that of the other regions based on different softwares'. ISSR marker is also highly important for detecting the genetic diversities of black cumin plant according to this study.

## Conflict of interests

The Authors declare that they have no competing interests.

## ACKNOWLEDGMENT

Our sincere thanks to the Ethiopian Institute of Biodiversity (EIB) for providing germplasms with full passport data of N. sativa assembled from Ethiopia.

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academicJournals

Vol. 14(18), pp. 1552-1563, 6 May, 2015
DOI: 10.5897/AJB2014.14379
Article Number: A050C1F52710
ISSN 1684-5315
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http://www.academicjournals.org/AJB

African Journal of Biotechnology

*Full Length Research Paper*

# Inter simple sequence repeat (ISSR) analysis of Ethiopian white lupine (*Lupinus albus* L.)

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Received 19 December, 2014; Accepted 28 April, 2015

**White lupine (*Lupinus albus* L.) collected from two zones (West Gojjam and Awi) of Amhara region and one zone (Metekel) of Benishangul - Gumuz regional state of Ethiopia were studied using inter simple sequence repeat (ISSR) markers in an attempt to assess the genetic diversity. Four ISSR primers of which three were dinucleotide repeats and one, a penta nucleotide repeat amplified a total of 39 clear and reproducible bands. Both unweighted pair- group method with arithmetic average (UPGMA) phenograms and a neighbor joining (NJ) trees were constructed for the individuals and populations using Jaccard's similarity coefficient. The dendrogram clearly indicated four distinct groups/populations based on the area of origin. The principal coordinates (PCO) analysis also recovered UPGMA and neighbor joining tree groups, although Amhara region white lupine were intermixed with each other. The genetic diversity among white lupine population considered in the present study indicated that Merawi was the highest (0.223) followed by Addis Kidam, Sekela and Wembera with genetic diversity of 0.198, 0.189 and 0.167, respectively. Generally, Amhara region white lupine (0.203) population shows higher genetic diversity than white lupine population of B-Gumuz region (0.167). Analysis of molecular variance (AMOVA) in both grouping and without grouping revealed larger genetic diversity within the populations (74.6%) than among populations (25.4%). Shannon's diversity index also confirmed the existence of higher genetic diversity in Amhara region lupine populations than in Benishangul-Gumuz. Furthermore AMOVA demonstrated highly significant (P = 0.00) genetic differences among populations within groups, among groups and within populations. Of the total variation, 64.64% was attributable to within populations, 27.23% to among groups and the least, 8.13% to among populations within groups. Generally, on the basis of samples of 39 bands in the four populations, ISSR was able to reveal moderate to high levels of genetic diversity within and among Ethiopian white lupine population.**

**Key words:** Amhara, Benishangul - Gumuz, Ethiopia, genetic diversity, ISSR, white lupine.

## INTRODUCTION

The genus *Lupinus* belongs to the family Fabaceae (Leguminosae) subfamily Papilionoideae tribe Genisteae (Gladstons, 1998). Originally, the name lupine was derived from the Latin "*Lupus*" (wolf) because it was

thought that they deplete soil nutrients, but the opposite is true as they are legumes and replenish the soil by nitrogen fixation (Christou, 1992). The Ancient Greeks used to call lupine as Thermes, while it is called Turmus in most Arab countries and India (Belteky and Kovacs, 1984). The species of the genus *Lupinus* are distributed in two centers of origin; one in the Mediterranean basin and the other extends through South America (Cowling et al., 2000). There are over 300 species in the genus *Lupinus* but only five are cultivated: white lupine (*Lupinu salbus),* blue lupine (*Lupinus angustifolius*), yellow lupine (*Lupinus luteus*), variable lupine (*Lupinus mutablis*) and garden lupine (*Lupinus polyphillus*). Only four of these have gained agricultural importance. These are *L. albus*, *L. angustifolius* and *L. luteus* of the "Old World" lupine species, and one "New World" species namely *L. mutabilis.* The first three species originated in the Mediterranean area, while *L. mutabilis* belongs to South America. The genus is comprised of geographically separated centers of diversity (Hondelmann, 1984). Borek et al. (2009) reported that the main fatty acid in yellow lupine cotyledons was linoleic acid; in white lupine it was oleic acid, and in Andean lupine, it was both linoleic and oleic acids. The white lupine is an old world species mainly distributed around the Mediterranean and along the Nile valley, where it has been traditionally cultivated for several thousands of years. These cultivated populations constitute the genetic resources of the species. Based on the modification in agricultural practices, genetic erosion in these areas has been extremely rapid (Huyghe, 1997).

Lupines have digitate leaves. The inflorescence is a raceme and the plant height can be up to 1.5 m. The flowers are quite distinctive and mainly self-pollinating but can be occasionally pollinated by bees. The inflorescence bearing the flowers varies 10 to 60 cm long depending on the species. Depending on the species the flowers can be white, pink or blue and are 12 to 16 mm in size. The seed pods are green and silky, up to 13 cm long and often constricted between the seed (Clapham et al., 1987). Lupines are cultivated in the world for three main uses: (1) for human nutrition because of their high protein and oil contents; (2) as green manure contributing to improved soil structure, with an increase of the organic matter content and through nitrogen and phosphorus accumulation in poor sandy soils; and (3) as ruminant feed either as green forage in the areas of traditional cultivation or, more often, as grains introduced as protein supplements in the diets of ruminants (Faluyi et al., 2000).

White lupine is adapted to well drained, light to medium textured, moderately acidic or neutral soils with a pH range of 4.5 to 7.5 (Gladstones, 1998). Currently, it is a minor crop in central Europe, while it is being widely grown in America. Lupine is also a traditional pulse crop, grown around the Mediterranean and in the Nile valley, extending to Sudan and Ethiopia. It is also grown in some parts of South-eastern and Southern Africa (Jansen, 2006). The white lupine in Ethiopia is locally known as “Gibto”. It is produced by small holder subsistent farmers mainly in two regional states of Ethiopia; Amhara and Benishangul-Gumuz, the former being the largest producer. It is grown in elevations ranging between 1500 to 3000 m.a.s.l. In the main production season (June to December) of the year 2008, a total of 17, 241 tons of lupines, with a mean productivity of 0.84 t/ha, were produced in these two major lupine producing regional states (ECSA, 2009). According to Francis (1999), the white lupine variety grown in North-western Ethiopia is bitter due to its high alkaloid content. He also reported that, though the variety is bitter, it is relatively non-shattering, high yielding and most importantly resistant to lupine anthracnose disease which is currently a problem for the cultivation of white lupine in some parts of Western Australia and Europe. Lupine production by small holder farmers in Ethiopia is targeted for its grain and soil fertility maintenance values. Its grain is used as snack and for the preparation of local alcoholic drink, Areki and local sauce called Shiro (made of lupine flour) (Likawent et al., 2010). White lupine (2n = 50) and other *Lupinus* species have been fully domesticated only recently when compared with most other crops. Genetic variability especially for extreme temperatures and drought tolerance, and disease and insect resistance in the cultivated germplasm is very low (Raman et al., 2008).

The genetic diversity of white lupine and other species of *Lupinus* have been characterized using morphological and agronomical attributes (Gonzalez et al., 2007), isozymes/proteins (Vaz et al., 2004) and molecular markers including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and inter simple sequence repeats (ISSR). Assessment of genetic diversity on the basis of morphological traits is not very reliable, as it may be influenced by the environment, and the number of traits with known inheritance is small. Molecular markers have the distinct advantages of being independent of climatic variables

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**Abbreviations: ISSR,** Inter simple sequence repeats; **UPGMA,** unweighted pair- group method with arithmetic average; **NJ,** neighbor joining; **PCO,** principal coordinates; **AMOVA,** analysis of molecular variance; **RAPD,** random amplified polymorphic DNA; **AFLP,** amplified fragment length polymorphism.

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**Table 1.** List of *Lupinus albus* populations with regions and site coordinates.

| Populationcode | Region | Zone, District (Wereda) | Sample size | Average Longitude/Latitude | Average altitude (m.a.s.l) |
|---|---|---|---|---|---|
| Mrw 1-10 | Amh | W.Gojjam, Mecha | 10 | 11° 38' 82" N/37° 14' 85"E. | 2059 |
| Skl 11-20 | Amh | W.Gojjam, Gish Abay | 10 | 10° 59' 07"N/37° 12'10" E. | 2736 |
| Adk 22-30 | Amh | Awi, Fagita Lekoma | 09 | 11° 5' 20" N/36° 54' 16" E. | 2494 |
| Wbr 31-40 | BG | Metekel, Wembera | 10 | 10° 34' 60" N/35° 47' 60" E | 2539 |
| Total | | | 39 | | |

Mrw - Merawi, Skl - Sekela, Adk - Addis Kidam, Wbr - Wembera, Amh - Amhara and BG - Benishangul-Gumuz.

**Table 2.** List of primers used for the analysis.

| Primer code | Annealing T° (°C) | Primer sequence | Amplification pattern |
|---|---|---|---|
| UBC - 810 | 45 | $(GA)_8T$ | Very Good |
| UBC - 818 | 48 | $(CA)_8G$ | Poor |
| UBC - 824 | 48 | $(TC)_8G$ | Poor |
| UBC - 834 | 45 | $(AG)_8YT$ | Excellent |
| UBC - 835 | 48 | $(AG)_8YC$ | Poor |
| UBC - 844 | 48 | $(CT)_8RC$ | Very Good |
| UBC - 860 | 45 | $(CT)_8RA$ | Poor |
| UBC - 880 | 45 | $(GGAGA)_3$ | Very Good |

Y = Pyramidine (C or T) and R = Purine (A or G).

and very numerous (Raman et al., 2014), and one of the most convenient and popular methods to identify and study of intraspecific genetic polymorphism is the ISSR-PCR method (Grishin et al., 2011). Although, lupine has immense potential for feed, food and soil fertility maintenance perspective (Anokhina et al., 2012) the Ethiopian lupine cultivation, genetic improvement and utilization remains far behind the other pulse crops. The unavailability of detailed information about the production system, current uses, genetic status and potentials, etc. are limitations of the Ethiopian white lupine. Therefore, this study is aimed at detailing the genetic diversity of an underutilized indigenous crop plant as revealed by a DNA marker.

## MATERIALS AND METHODS

### Plant materials and sampling strategy

A total of 39 individual plants of *L. albus* L. representing four populations were collected from four districts or weredas each from Wembera in Benishangul-Gumuz (Metekel zone), and Merawi and Sekela (West Gojjam zone) and Addis Kidam (Awi zone) of Amhara reginal state. Lupine plants growing on an individual farmers plot of land were considered as a single population. Hence, young leaves from individual lupine plants were selected randomely with approximately 10 m distance from each and collected separetely. The four populations are indicated in Table 1 along with altitude and site coordinates of each of the locality they are collected from.

### DNA extraction

Genomic DNA extraction was done based on the method described in Borsch et al. (2003) which involves a modified triple CTAB extraction method to yield optimal amounts of DNA. All DNA extractions were carried from silica gel dried leaf sample and ground by liquid nitrogen at Genetics Research Laboratory, Department of Biology Addis Ababa University (AAU).

### Genomic test gel and electrophoresis

An agarose gel (100 ml, 1xTBE and 0.98 g agarose) was prepared and 2 µl of each genomic DNA samples mixed with 6 µl loading dye (1X bromophenol blue) was loaded on the gel and electrophoresed at constant voltage of 80 V for 45 min. The gel was stained for 30 min with 50 µl ethidium bromide (10 mg/ml) after well mixed with 450 ml distilled water. Then, it was destained/ washed for 30 min with distilled water. Gel picture was taken under UV transilluminator by BiodocAnalyse 2.0 with digital canon camera. From the two extractions following the protocol given by Borsch et al. (2003), those with high band intensity and less smear were selected for PCR, and this was commonly the case for the second extractions.

### Primer selection and optimization

A total of eight ISSR primers used by Talhinhas et al. (2003) and Mustafa et al. (2009) were used for the initial testing of polymorphism and reproducibility of PCR products. DNA from three individual plants was selected from each population to screen the primers. Based on polymorphism and reproducibility, four primers were selected for the study (Table 2).

### PCR amplification and electrophoresis

The polymerase chain reaction was done using Biometra 2000 T3 Thermo cycler. PCR amplification was carried out in a 25 µl total reaction mixture containing 1 µl template DNA, 13.2 µl $ddH_20$, 5.6 µl

**Table 3.** Selected ISSR primers with their amplification and banding pattern.

| ISSR primer | Repeat motif | Amplification pattern | Number of scorable bands |
|---|---|---|---|
| UBC – 810 | $(GA)_8T$ | Very Good | 10 |
| UBC – 834 | $(AG)_8YT$ | Excellent | 12 |
| UBC – 844 | $(CT)_8RC$ | Very Good | 8 |
| UBC – 880 | $(GGAGA)_3$ | Very Good | 9 |
| Total | | | 39 |

dNTP (1.25 mM), 2.6 µl PCR buffer (10xThermopol reaction buffer), 2.0 µl $MgCl_2$ (2 mM), 0.4 µl primer (20 pmol/µl) and 0.2 µl Taq Polymerase (5 U/µl). The amplification program was 4 min preheating and initial denaturation at 94°C, then 39 x 15 s at 94°C, 1 min primer annealing at (45/48°C) based on primers used, 1.30 min extension at 72°C. The final extension for 7 min at 72°C followed. The PCR products were also stored at 4°C until loaded on gel for electrophoresis. An agarose gel (1.67 gm agarose with 100 ml 1xTBE) was prepared and 8 µl amplification product of each sample with 2 µl loading dye (bromophenole blue 6X) was loaded on gel. DNA seizer of 1 kb DNA ladder was used to estimate molecular weight of ISSR fragments. The electrophoresis was done for 2 to 3 h at constant voltage of 100 V. The gel was stained with ethidium bromide (10 mg/ml) for 30 min and destained for a further 30 min. Gel picture was taken with Biometra Biodoc analyzer.

#### Data scoring and analysis

Each bands that was amplified using ISSR primers, was treated as a unit character and scored as '0' for absence, '1' for presence and '?' for missing or ambiguous. POPGENE version1.32 software (Yeh et al., 1999) was used to calculate genetic diversity for each population as number of polymorphic loci, percent polymorphism, gene diversity and Shannon diversity index. Shannon–Weaver diversity index (H) was calculated as H= -Σpilog2pi; where, pi is the frequency of a given band for each population (Lewontin, 1972). Shannon's index of diversity was used to measure the total diversity (Hsp) for the species as well as the mean diversity per population (Hpop). The proportion of diversity within and between populations was then calculated as Hpop/Hsp and (1-Hpop/Hsp), respectively. Analysis of molecular variance (AMOVA) (Excoffier et al., 2006) was used to calculate variation among and within population using Areliquin version 3.01 (Excoffier et al., 2006). NTSYS- pc version 2.02 (Rohlf, 2000) and Free Tree 0.9.1.50 (Pavlicek et al., 1999) softwares were used to calculate Jaccard's similarity coefficient which is calculated as:

$$S_{ij} = \frac{a}{a+b+c}$$

Where, "a" is the total number of bands shared between individuals i and j, "b" is the total number of bands present in individual i but not in individual j and "c" is the total numbers of bands present in individual j but not in individual i.

NTSYS- pc version 2.02 Rohlf (2000) was used to generate the unweighted pair group method with arithmetic mean (UPGMA) phenogram to analyze and compare the individual genotypes. The NJ method (Saitou and Nei, 1987; Studier and Keppler, 1988) was used to compare individual genotypes and evaluate patterns of genotype clustering using Free Tree 0.9.1.50 Software (Pavlicek et al., 1999). The major difference between the two algorithms is that UPGMA assumes equal rates of evolution (molecular clock assumption) along all branches, whereas neighbor joining assume variations in the rate of change (Saitou and Nei, 1987; Studier and Keppler, 1988; Nei and Kumar, 2000; Lan and Reeves, 2002). To further examine the patterns of variation among individual samples, a principal coordinate analysis (PCO) was performed based on Jaccard's coefficient (Jaccard, 1908). The calculation of Jaccard's coefficient was made with PAST software version 1.18 (Hammer et al., 2001). The first three axes were later used to plot with STATISTICA version 6.0 software (Hammer et al., 2001; Statistica Soft, Inc. 2001).

## RESULTS AND DISCUSSION

### Banding patterns and ISSR primers

Out of the eight primers tested initially, four of them gave relatively clear banding pattern and they were selected and used in this study (Table 3). The size of the fragments amplified using the four primers were in the range of 450 bp to 4 kb. A total of 39 fragments were amplified by the four ISSR primers of which 32 (82%) were polymorphic. The highest number of bands (12) was recorded for primer UBC - 834 followed by UBC 810 and UBC 880 which generated 10 and 9 scorable bands respectively. The least number of bands (8) were amplified by primer UBC - 844 (Figures 1 and 2).

### Polymorphism

The number of polymorphic loci ranges from 5 for UBC-844 to 10 for UBC-834, where all are SSR with di-nucleotide repeat motif (Table 4). The only penta-nucleotide repeat primer UBC-880 and di-nucleotide UBC-810 showed the polymorphism of 8 and 9. Considering the percent polymorphism, UBC-844 showed the least polymorphism with 62.5%, while UBC-810 showed the highest with 100% polymorphism.

Among all the populations, studied Merawi and Addis Kidam from Amhara were found to show higher percentage polymorphism with 53.85 and 48.72%. This might be due to transfer of genes by effectors such as wind, insect, birds and or human (seedling movement) e.t.c. since these two weredas (Merawi and Addis Kidam) are near each other and in the main road of Bahir - Dar to Addis Ababa. Sekela of Amhara region has a percent polymorphism of 46.15%. The least percent

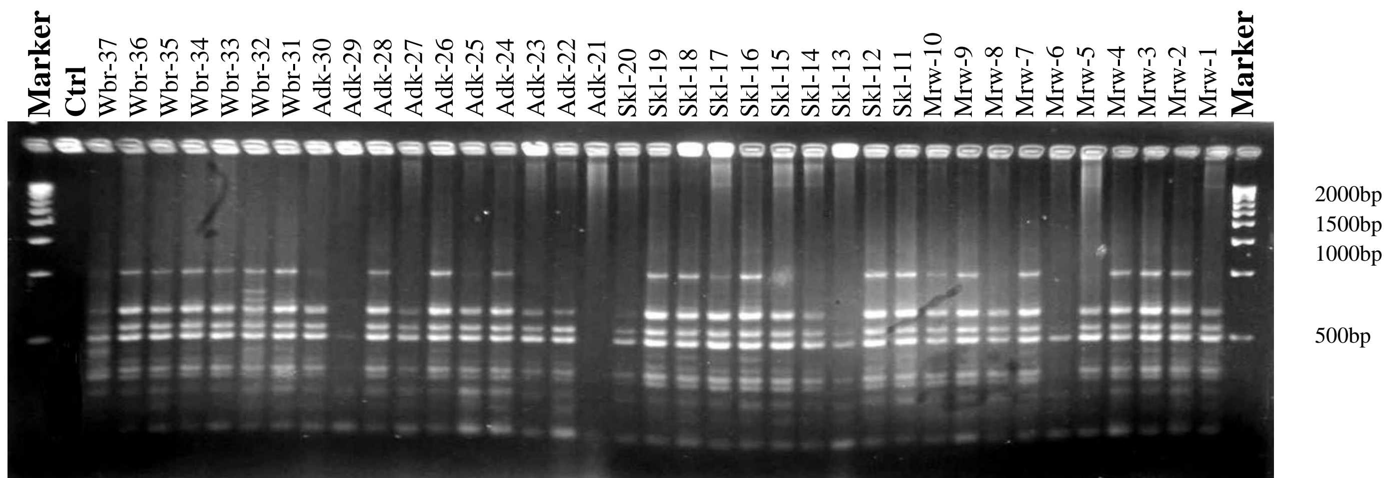


**Figure 1.** Banding pattern of lupine DNA samples by primer UBC - 834.

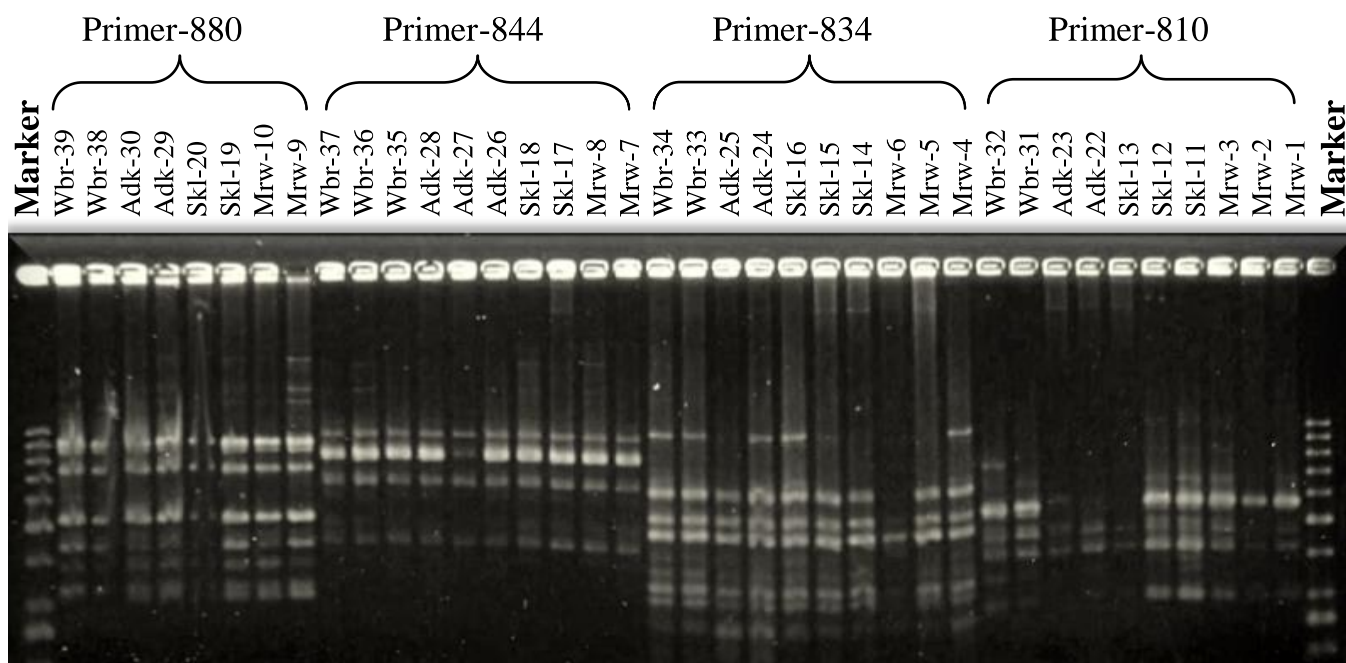


**Figure 2.** Banding pattern of lupine DNA samples by primers UBC - 810, UBC - 834, UBC - 844 and UBC - 880.

polymorphism was observed in Wembera of B-Gumz region population with 43.59%. Generally, Amhara region white lupine population has higher percent polymorphism (49.57%) than that of B-Gumuz region (43.59%) (Table 4).

## Genetic diversity

Among white lupine populations considered in the present study, genetic diversity was higher for Merawi population (0.223) followed by those of Addis Kidam, Sekela and Wembera populations with genetic diversity of 0.198, 0.189 and 0.167, respectively. From this work it seems that, Amhara region white lupine (0.203) population shows higher genetic diversity than white lupine population of B-Gumuz region (0.167) (Table 4). This might be also due to transfer of genes by effectors like that of the reason for polymorphism.

## Partitions of genetic variation

Levels variations can be attributed to within and between

**Table 4.** Number of polymorphic loci (NPL), percent polymorphism (PP) and genetic diversity (GD) of white lupine with each population and all primers.

| Population | With all primers | | |
|---|---|---|---|
| | NPL | PP (%) | GD |
| **Amhara Region** | | | |
| Merawi | 21 | 53.85 | 0.223 |
| Sekela | 18 | 46.15 | 0.189 |
| Addis Kidam | 19 | 48.72 | 0.198 |
| Sum | 58 | 148.72 | 0.61 |
| Average | 19.3 | 49.57 | 0.203 |
| **B-Gumuz Region** | | | |
| Wembera | 17 | 43.59 | 0.167 |
| Over all | 75 | 192.31 | 0.777 |
| Average over all | 18.75 | 48.078 | 0.194 |
| **For individual primers** | | | |
| UBC – 810 | 9 | 100 | 0.301 |
| UBC – 834 | 10 | 83.33 | 0.319 |
| UBC – 844 | 5 | 62.5 | 0.140 |
| UBC – 880 | 8 | 80.0 | 0.233 |
| Sum | 32 | 325.83 | 0.993 |
| Average | 8 | 81.46 | 0.248 |

**Table 5.** Shannon's diversity index within and among white lupine populations with di and penta-nucleotide primers.

| Population | Shannon's diversity index (H) | | Over all H |
|---|---|---|---|
| | Di-nucleotide repeat primers | Penta-nucleotide repeat primers | |
| Merawi | 0.291 | 0.389 | 0.324 |
| Sekela | 0.308 | 0.131 | 0.272 |
| Addis Kidam | 0.456 | 0.286 | 0.289 |
| Wembera | 0.309 | 0.111 | 0.247 |
| Hpopn | 0.341 | 0.229 | 0.283 |
| Hsp | 0.405 | 0.405 | 0.405 |
| Hpopn/Hsp | 0.842 | 0.565 | 0.698 |
| 1- Hpopn/Hsp | 0.158 | 0.435 | 0.302 |

Hpopn = mean genetic variation for population, Hsp = mean genetic variation for the entire data, Hpopn/Hsp = proportion of genetic variations within white lupine populations and 1-Hpopn/Hsp = proportion of genetic variations between white lupine populations.

population components. Shannon's diversity index and AMOVA were used to partition the existing genetic variation in to different components.

## Shannon Weaver's diversity indices

The overall analysis with both di and penta-nucleotide primers indicated that the white lupines collected from Merawi were found to be more diversified compared to the rest of the populations collected from other weredas of Amhara and B-Gumuz region as shown in Table 5. Generally, Amhara region white lupine populations show higher Shannon`s diversity indices than the population of B-Gumuz region. The partitioning of the mean Shannon weaver diversity index for the species revealed that white

**Table 6.** AMOVA of white lupine populations, A; without grouping. B; with groups.

| Source of variation | d.f | Sum of squares | Variance components | Percentage of variation | Fixation indices | P |
|---|---|---|---|---|---|---|
| **A** | | | | | | |
| Among populations | 3 | 26.630 | 0.69986 | 25.38 | 0.25382 | 0.00 |
| Within populations | 35 | 72.011 | 2.05746 | 74.62 | | 0.00 |
| Total | 38 | 98.641 | 2.75732 | | | |

**B**

| Source of variation | d.f | Sum of squares | Variance components | Percentage of variation | P |
|---|---|---|---|---|---|
| Among groups | 1 | 17.517 | 0.86686 | 27.23 | 0.00 |
| Among populations within groups | 2 | 9.113 | 0.25883 | 8.13 | 0.00 |
| Within populations | 35 | 72.011 | 2.05746 | 64.64 | 0.00 |
| Total | 38 | 98.641 | 3.18315 | | |

lupines are more variable among individuals of a population (0.698) than among the different populations (0.302). This result is similar with the work of Solomon (2007), who works in genetic diversity analysis of the wild *Coffea arabic*a L., populations from Harenna Forest, Bale Mountains of Ethiopia, using inter simple sequence repeats (ISSR) and Tesfaye (2006) Genetic diversity of wild *C. arabica* L., populations in Ethiopia as a contribution to conservation and use planning. The mean genetic diversity for populations was higher with di-nucleotide repeat primers as compared to penta-nucleotide repeat primers but equal value for mean genetic variation for the entire data (Table 5).

## Analysis of molecular variance (AMOVA)

Analysis of molecular variance was carried out in two phases; one was done using the populations grouped into Amhara and B-Gumuz and the other was done for the entire populations (that is, using the four populations as it is without grouping) over all loci by considering them as one geographic region. The analysis was carried out by computation of the distance between "haplotypes", each individual's data pattern as one "haplotype" and computing variance components for each level (Excoffier et al., 2006). Partitioning of genetic diversity by analysis of molecular variance using grouped populations (Table 6B) revealed that out of the total genetic diversity, most of the ISSR diversity was distributed between individual plants within the populations (64.6%), with the remaining diversity being distributed among populations within groups (8.13%) and among groups (27.23%). Similarly, partitioning of genetic diversity by analysis of molecular variance without grouping populations revealed that out of the total genetic diversity, most of the ISSR diversity is due to differences between individual plants within the populations (74.6%), while the remaining is due to differences among populations (25.4%) (Table 6A). In both cases, the results of AMOVA revealed the same patterns of genetic diversity and indicated larger genetic diversity within the populations rather than among populations. This result is similar with the work of Solomon (2007) Tesfaye (2006), on wild *C. arabic*a L.

## Genetic similarity

In Figure 3, it is indicated that an UPGMA dendrogram of white lupine populations was constructed based on Jaccard's similarity coefficients (Table 7). High similarity was observed between Sekela and Merawi white lupine populations (0.741) followed by the value between Addis Kidam and Merawi (0.734). The least similarity was observed between Wembera and Merawi. Sekela and Merawi which were collected from geographically close weredas of west Gojjam zone of Amhara region form their own group confirming the correlation of genetic distance with geographic distance. The Wembera population shares relatively smaller similarity values with all the populations from the Amhara region. Thus, the Wembera lupine population had similarity values of 0.715, 0.703 and 0.679 with the population of Addis Kidam, Sekela and Merawi, respectively. As it is shown on the UPGMA tree, Wembera is isolated from the other three and this is also correlated with the geographic distance separating these populations.

## Cluster analysis

Jaccard's similarity coefficients were also used to construct UPGMA and NJ dendrograms for 39 individuals based on the bands obtained with the four primers (Figure 5).

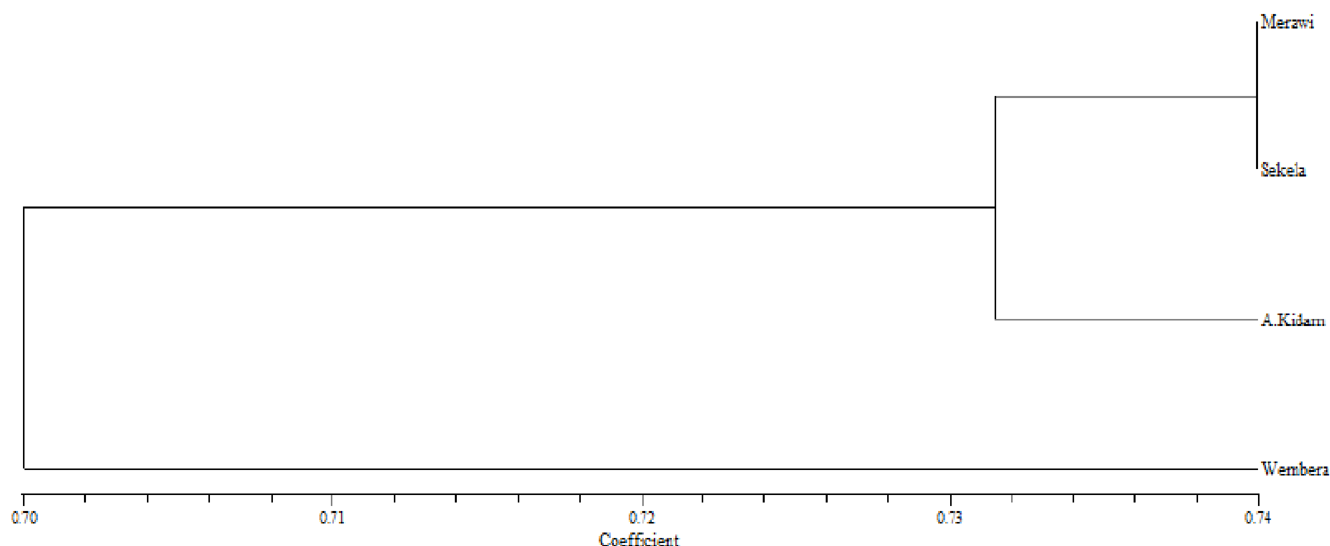


**Figure 3.** UPGMA based dendrogram for 4 white lupine populations using 4 ISSR primers.

**Table 7.** Similarity matrix for Jaccard's coefficients for 4 white lupine populations based on the bands obtained with ISSR primers.

| Population | Merawi | Sekela | Addis Kidam | Wembera |
|---|---|---|---|---|
| Merawi | 1.000 | | | |
| Sekela | 0.741 | 1.000 | | |
| Addis Kidam | 0.734 | 0.730 | 1.000 | |
| Wembera | 0.679 | 0.703 | 0.715 | 1.000 |

Accordingly, individuals collected from Wembera wereda of B-Gumuz region located farther west than the other populations tend to form strong separate group in both UPGMA and NJ dendrogram. However, populations collected from Amhara region were observed to form moderate grouping based on their place of origin (Figure 4). Generally, both trees recovered almost the same tree topology with similar groupings, although few individuals appeared to escape from groups in case of NJ analysis.

## Conclusion

Currently, a number of molecular markers have been widely used to study diversity in many plants (Karp et al., 1997). Given the proliferation of molecular markers, a comparison between the markers seems highly inevitable on the basis of study objectives and the nature of the markers. Of the many desired qualities of molecular markers, automation (PCR-based), polymorphisms and reproducibility are the highly demanded features of the molecular techniques to be used in the intraregional diversity analysis. ISSR markers are thus one of the molecular markers that have these characteristics to study variability in different crops (Zietkiewicz et al., 1994, Wolf and Liston, 1998). ISSR markers are observed to be highly variable within the species and reveal many more polymorphisms since they use longer primers that allow more stringent annealing temperatures (Hillis et al., 1996). Moreover, this marker observed to be very useful in detecting genetic diversity and population structure of Coffee (Aga, 2005); (Tesfaye, 2006), Tef (Assefa, 2003), and rice (Gezahegn, 2007) collected from all over Ethiopia.

In this study, also the ISSR markers observed to be an appropriate molecular marker for generating the detailed intraspecific genetic diversity data to evaluate extent and distribution of genetic diversity within and among *L. albus L.* Out of the total 39 scorable bands produced with the total of four; 3 di- and 1 penta-nucleotides, 32 bands were polymorphic. In terms of number of polymorphic fragment detected and percentage of polymorphic loci, per class of primer, di-nucleotides were found to be superior. In general, the detection of high levels of polymorphisms makes ISSR analysis with di-nucleotides primers a powerful technique for measuring the genetic diversity in white lupine. Among white lupine populations considered in the present study, Merawi has higher genetic diversity (0.223) than Sekela (0.186), Addis Kidam (0.161) and Wembera (0.167). Generally, Amhara region white lupine (0.203) populations show higher genetic diversity than white lupine population of B-Gumuz region.

Partitioning of genetic diversity by analysis of molecular variance using grouped populations revealed that out of the total genetic diversity, most of the ISSR diversity was distributed between individual plants within the populations (64.6%), with the remaining diversity being distributed among populations within groups (8.13%) and

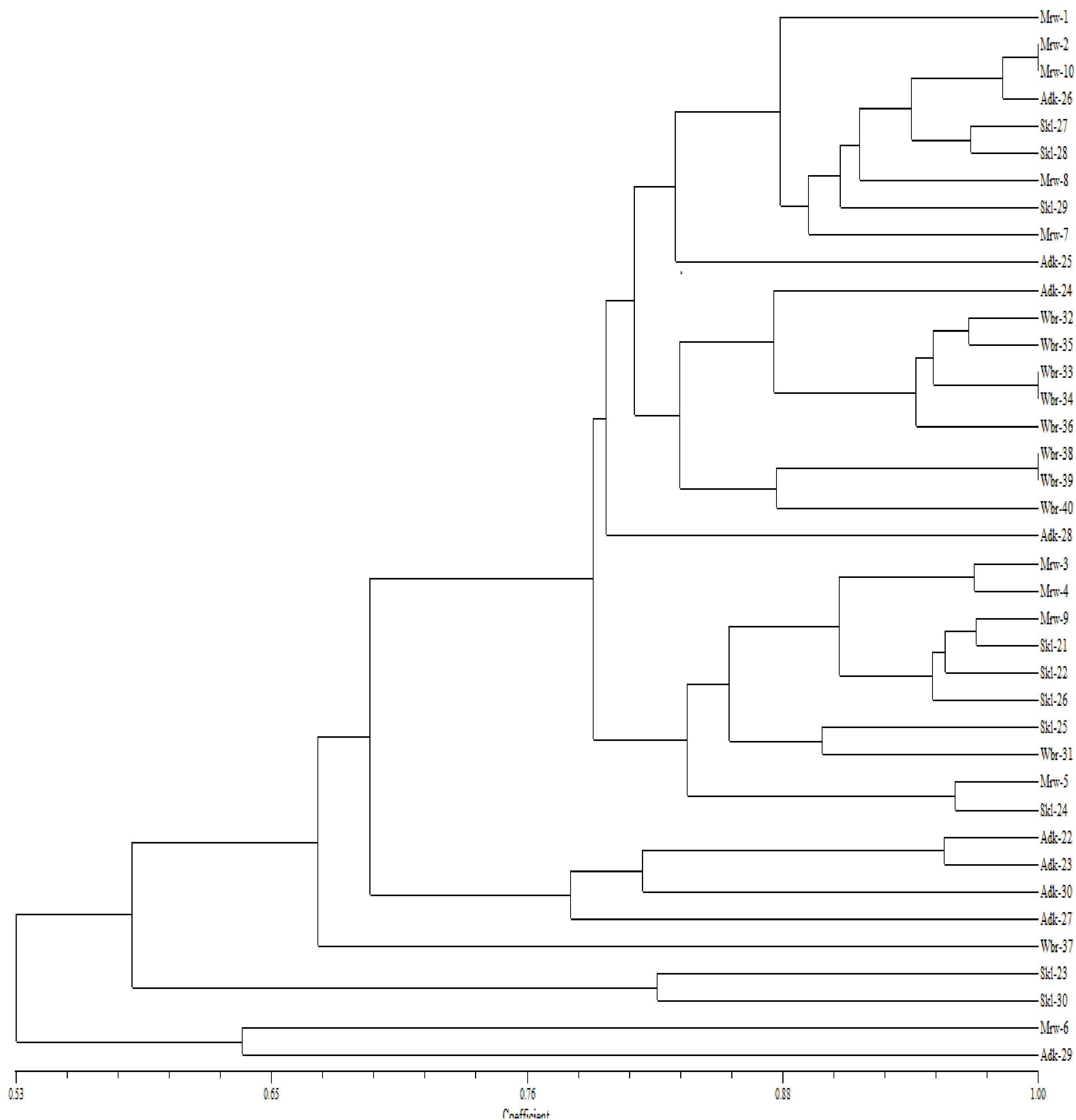


**Figure 4.** UPGMA dendrogram depicting clustering patterns for 39 individuals of white lupine based on Jaccard's similarity coefficient.

among groups (27.23%). Similarly, partitioning of genetic diversity by analysis of molecular variance without grouping populations revealed that out of the total genetic diversity, most of the ISSR diversity is due to differences between individual plants within the populations (74.6%), while the remaining is due to differences among populations (25.4%). In both cases, the results of analysis of molecular variance revealed the same pattern of genetic diversity and supports the larger genetic diversity found within the populations rather than among populations Based on Jaccard's coefficients of similarity, high genetic similarity was observed between Sekela and

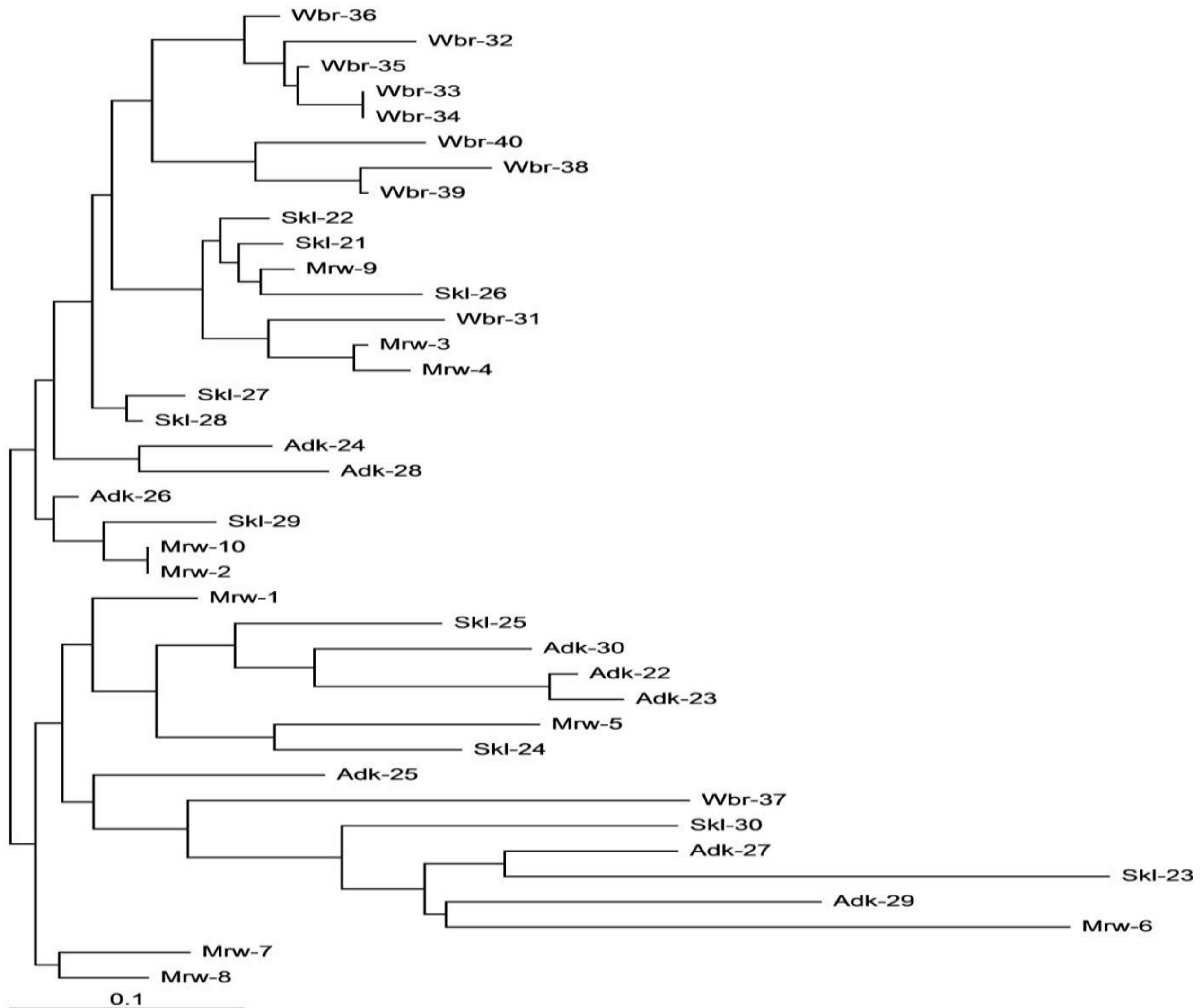


**Figure 5.** NJ analysis of 39 individuals based on Jaccard's similarity coefficient.

Merawi white lupine populations (0.741) followed by the value between Addis Kidam and Merawi (0.734). The least similarity was observed between Wembera populations with Merawi populations (0.679). The Wembera population shares relatively smaller similarity values with all the populations from the Amhara region. Thus, the Wembera lupine population had similarity values of 0.715, 0.703 and 0.679 with the population of Addis Kidam, Sekela and Merawi, respectively.

In the present study, four ISSR primers; 3 di- and 1 penta- nucleotides were employed. They were able to reveal that genetic diversity ranged from moderate to high levels and identified the highly diverse and least diverse populations in Ethiopian white lupine collected from Amhara and B-Gumuz region. While, high genetic diversity was observed between two regions, moderate levels of variation were shown in Amhara region populations. The Shannon's diversity index also confirmed the existence of higher diversity in two regions population and genetic similarity based on Jaccard's coefficients of similarity was observed high value between Sekela and Merawi white lupine populations (0.741) followed by white lupine populations from Addis Kidam and Merawi (0.734), the least similarity was found between Wembera population with Merawi and Sekela having the value of 0.679 and 0.703. Though a limited number of ISSR markers were used in the study, the results confirm that ISSR markers are efficient in detecting polymorphism within and among populations of white lupine found in close geographic proximity. Until the present day information available on the reproductive biology of *L. albus* L., suggested that it is a predominantly self-pollinating plant. However, the result of this study might be attributed to two reasons: one against and the other in favor of the self-pollinating nature of the *L. albus* plant. In the former case, the result obtained could be

accounted to mixed type of mating, typical of plant species, in which there is a gene flow, and thus there might be moderate gene flow among the local populations by effectors such as wind, insect, human (seedling movement) and birds. The other is that they might have preferential or diverse adaptive genes that are not fixed through self-pollination until the present day.

However, this study used a small sample size, geographic range and limited primers. Therefore, to find clear patterns of diversity for the whole country and reach a sound conclusion, further studies should be conducted with large sample sizes and geographic range using many ISSR primers.

## Conflict of interests

The authors did not declare any conflict of interest.

## ACKNOWLEDGEMENTS

This work was financially supported by school of graduate studies of Haramaya University. Training on common molecular marker techniques and their application in plant breeding and conservation of plant genetic resources and laboratory work was provided and supported by Addis Ababa University. We thank all the institution and individuals from our bottom of heart.

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Vol. 14(18), pp. 1564-1575, 6 May, 2015
DOI: 10.5897/AJB2014.14320
Article Number: EB941A052712
ISSN 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

# Genotype x environment interaction and stability analysis for yield and yield related traits of Kabuli-type Chickpea (*Cicer arietinum* L.) in Ethiopia

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Received 14 November, 2014; Accepted 28 April, 2015

**Chickpea is the major pulse crop cultivated in Ethiopia. However, its production is constrained due to genotype instability and environmental variability. This research was carried out to examine the magnitude of environmental effect on yield of chickpea genotypes and to investigate the stability and adaptability of genotypes under different agro-ecologies. Seventeen (17) genotypes were evaluated in randomized complete block design (RCBD) with four replications in five locations. Various stability indices were used to assess stability and genotype by environment performances. Combined analysis of variance (ANOVA) for yield and yield components revealed highly significant (P≤0.01) differences for genotypes, environments and their interaction. The significant interaction showed genotypes respond differently across environments. At Akaki, Chefe Donsa, Debre Zeit, Dembia and Haramaya, top performing genotype were DZ-2012-CK-0001 (2933 kg/ha), Arerti (3219 kg/ha), Arerti (3560 kg/ha) DZ-2012-CK-0013 (2675 kg/ha) and Arerti (2019 kg/ha), respectively. The first two PCs explained 74.45% of the variance. Based on ASV value, DZ-2012-CK-0002 were most stable genotypes. As per AMMI biplot, Arerti and DZ-10-4 were most widely adapted genotypes. Dembia and Haramaya were most discriminative environments for genotypes. Debre Zeit and Chefe Donsa were favorable environment for genotype. Genotypes DZ-2012-CK-0004, DZ-2012-CK-0010, DZ-2012-CK-0013, DZ-2012-CK-0007 and DZ-10-4 are recommendable to Akaki, Chefe Donsa, Debre Zeit, Dembia and Haramya, respectively.**

**Key words:** AMMI, ASV, clustering, phenologic traits, Kabuli, univariate statistics.

## INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a cool season legume that ranks third among the pulses in area and production worldwide. It is grown on around 1.1 million hectare with 9 metric tons global production (Babar et al., 2009). The

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**Abbreviations: E,** Environments; **G,** genotypes; **RCBD,** randomized complete block design.

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**Table 1.** Geographic and environmental conditions of experimental area.

| Trial site | Soil type | Altitude (masl) | Rainfall average (mm) | Temperature (°C) | | Geographical position | |
|---|---|---|---|---|---|---|---|
| | | | | Min | Max | Latitude (N) | Longitude (E) |
| Akaki | Vertisol | 2120 | 1055 | 10.36 | 22.3 | 08°52’ | 38°48’ |
| Chefe Donsa | Vertisol | 2450 | 950 | 10.5 | 23.2 | 08°52’ | 39°08’ |
| Dembia | Vertisol | 1885 | 1000 | 14.0 | 29.2 | 12°32830’ | 37°22065’ |
| Debre zeit | Vertisol | 1950 | 851 | 10.8 | 26.9 | 08°44’ | 38°58’ |
| Haramaya | Vertisol | 1980 | 780 | 15.8 | 24.3 | 9°26’ | 42°30’ |

Source: Debre Zeit Agricultural Research Center (2012).

**Table 2.** List of Kabuli-type chickpea genotypes included in the experiment

| Entry no. | Entry name | Entry no. | Entry name | Entry no. | Entry name |
|---|---|---|---|---|---|
| 1 | DZ-2012-CK-0001 | 8 | DZ-2012-CK-0008 | 15 | EJERE (SC) |
| 2 | DZ-2012-CK-0002 | 9 | DZ-2012-CK-0009 | 16 | HABRU (SC) |
| 3 | DZ-2012-CK-0003 | 10 | DZ-2012-CK-0010 | 17 | DZ-10-4 |
| 4 | DZ-2012-CK-0004 | 11 | DZ-2012-CK-0011 | | |
| 5 | DZ-2012-CK-0005 | 12 | DZ-2012-CK-0012 | | |
| 6 | DZ-2012-CK-0006 | 13 | DZ-2012-CK-0013 | | |
| 7 | DZ-2012-CK-0007 | 14 | ARERTI (SC) | | |

Kabuli type chickpeas are characterized by white-colored seed with ram’s head shape, thin seed coat, smooth seed surface, white flowers, and lack of anthocyanin pigmentation on the stem. The plant is medium to tall in height, with large leaflets and white flowers. When compared with Desi types, the Kabuli types have higher levels of sucrose and lower levels of fiber. The Kabuli types generally have large sized seeds and receive higher market price than Desi types (Gaur et al., 2010). Chickpea seeds are eaten fresh as green vegetables, parched, fried, roasted and boiled as snack food, sweet and condiments (Dawar et al., 2007). Environmental factors such as soil moisture, sowing time, fertility and temperature and day length have strong influence during various stages of plant growth (Bull et al., 1992). The environment is changing day-by-day and this implies that it is necessary to evaluate crop genotypes at different locations to assess their performances. One approach to improve the chickpea yield is to identify stable genotypes that perform consistently better under diverse environments (Ghulam et al., 2012). The performance of a genotype is not always the same in different locations as it is influenced by environmental factors. To assess yield stability among varieties, multi-location trials with appropriate stability analysis method is required. Differences in genotype stability and adaptability to environment can be qualitatively assessed using the biplot graphical representation that scatters the genotypes according to their principal component values (Vita et al., 2010).

In Ethiopia, there is no sufficient information on the genotype by environment interaction effects on yield and yield related traits of Kabuli-type chickpea. Therefore, the current research was undertaken to examine the magnitude of environmental effect on yield and yield related traits of Kabuli-type chickpea genotypes, to study the nature and extent of genotype by environment interaction on seed yield of Kabuli -chickpea genotypes and to investigate the stability and adaptability of the genotypes under different agro-ecological condition.

## MATERIALS AND METHODS

The experiment was conducted during the 2012/13 main cropping season at five locations representing various chickpea growing agro-ecologies of Ethiopia. The environments were Akaki, Chefe Donsa, Debre Zeit, Dembia and Haramaya. Thirteen (13) pipelines and four released Kabuli-type chickpea varieties were included in the study (Tables 1 and 2). The plant materials were obtained from Debre Zeit Agricultural Research Center. Planting of the genotypes was done in early mid August up to first week of September using randomized complete block design with four replications at each site under rain fed conditions. Each genotype was planted in six rows of 4 m row length and at 1.2 m width. A spacing of 30 cm row to row distance and 10 cm plant to plants were used on a plot size of 4.8 $m^2$. Fertilizer was not applied. Weeding and other management practice were done as required for each site. Data were recorded on days to 50% flowering, 90% physiological maturity, plant height, the number of pods per plant, the number of seeds per plant, 100-seed weight, biomass yield, grain yield, and harvest index.

**Table 3.** Mean sum of squares of yield and other traits from combined ANOVA of 17 Kabuli-type chickpea genotypes grown across five environments in Ethiopia.

| Source | DF | DF | DM | PPP | SPP | PHT | HSW | BM | HI | YLD |
|---|---|---|---|---|---|---|---|---|---|---|
| E | 4 | 3225** | 12608** | 7937** | 0.007** | 1432** | 401.1** | 29037069** | 11097** | 1504219** |
| G | 16 | 242.7** | 155** | 653** | 0.59** | 268** | 719.5** | 662654** | 258** | 84562** |
| G X E | 64 | 81.4** | 24** | 198** | 0.09** | 21** | 7.9** | 243679** | 63** | 28149** |
| Error | 240 | 6.7 | 7.8 | 29.6 | 0.01 | 12.3 | 2.3 | 51319 | 24.4 | 8138 |

DF = Days to flowering, DM = days to maturity, PPP = pod per plant, SPP = seed per pod, PHT = plant height, HSW = hundred seed weight, HI = harvest index and YLD = grain yield.

**Table 4.** Mean grain yield (kg/ha) of 17 Kabuli-type chickpea genotypes grown at five locations in Ethiopia.

| Genotype | Akaki | Chefe Donsa | Debre Zeit | Dembia | Haramaya | Mean YLD |
|---|---|---|---|---|---|---|
| DZ-2012-CK-0001 | 2933 | 2501 | 3115 | 1344 | 1628 | 2304 |
| DZ-2012-CK-0002 | 1940 | 2428 | 2604 | 1446 | 1443 | 1968 |
| DZ-2012-CK-0003 | 2452 | 2488 | 3304 | 1142 | 1248 | 2128 |
| DZ-2012-CK-0004 | 2552 | 2518 | 3106 | 1255 | 795 | 2042 |
| DZ-2012-CK-0005 | 2421 | 2346 | 2992 | 1655 | 1639 | 2210 |
| DZ-2012-CK-0006 | 1905 | 2035 | 2552 | 1337 | 1039 | 1774 |
| DZ-2012-CK-0007 | 1702 | 2265 | 2678 | 1485 | 1689 | 1774 |
| DZ-2012-CK-0008 | 2315 | 1940 | 3167 | 1795 | 1460 | 2135 |
| DZ-2012-CK-0009 | 2178 | 2408 | 2719 | 1617 | 1287 | 2042 |
| DZ-2012-CK-0010 | 1894 | 2046 | 3310 | 2062 | 1137 | 2090 |
| DZ-2012-CK-0011 | 1860 | 2120 | 2518 | 1023 | 1066 | 1718 |
| DZ-2012-CK-0012 | 1775 | 1879 | 3177 | 1348 | 1935 | 2023 |
| DZ-2012-CK-0013 | 2814 | 2733 | 2983 | 2675 | 1888 | 2635 |
| Arerti (SC) | 1433 | 3219 | 3560 | 1831 | 2019 | 2412 |
| Ejere (SC) | 1764 | 2477 | 2826 | 1370 | 1685 | 2025 |
| Habru (SC) | 2599 | 2849 | 3285 | 1419 | 1815 | 2393 |
| Dz-10-4 (LC) | 526 | 1814 | 2602 | 1387 | 1225 | 1510 |
| Means | 2063 | 2362 | 2970 | 1540 | 1469 | 2081 |
| CV (%) | 25 | 18 | 13 | 8 | 19 | 18 |
| LSD | 177 | 149 | 134 | 42 | 94 | 56 |

DF = Days to flowering, DM = days to maturity, PPP = pod per plant, SPP = seed per pod, PHT = plant height, HSW = hundred seed weight, HI = harvest index and YLD = grain yield, SC=standard check and LC = Local check.

#### Statistical analysis

Data were computed by using SAS 9.1.3 for analysis of variance, Genstat13$^{th}$ for biplot graph and Agrobase20 for stability analysis.

## RESULTS AND DISCUSSION

### Performance of Kabuli-type chickpea genotypes for yield

Performance trials have to be conducted in multiple environments because of the presence of GE. For the same reason, the analysis of genotype by environment data must start with the examination of the magnitude and nature of genotype by environmental interaction (Ezatollah et al., 2011). Yield and its components are polygenic traits and are strongly influenced by environment in chickpea. Significant variation was observed for grain yield in Kabuli chickpea genotypes. Similar finding were reported by Khan et al. (1987, 1988). Bartlett's test showed homogenous error variance for the grain yield and allowed to proceed further pooled analysis across environments.

The combined analysis of variance (Table 3) for grain yield exhibited significant (P≤0.01) effects of locations, genotypes and genotype by environment interaction, indicating differences in environments and the presence of genetic variability among genotypes. The presence of significant genotype by environment interaction in chickpea was reported by various authors (Singh et al., 1990; Bozoglu and Gulumser, 2000). The overall mean yield of the location varied from 1469 to 2970 kg/ha (Table 4) and thus, the five environments showed wide

**Table 5.** Mean values for yield related traits of Kabuli -type chickpea genotypes tested at five locations in Ethiopia (averaged over all genotypes).

| Environment | DF | DM | PPP | SPP | PHT | HSW | BM | HI | YLD |
|---|---|---|---|---|---|---|---|---|---|
| Akaki | 61 | 130 | 25 | 1.01 | 36 | 32 | 1071 | 48 | 2063 |
| Chefe Donsa | 56 | 143 | 30 | 1.01 | 40 | 29 | 1633 | 35 | 2362 |
| Debre Zeit | 54 | 108 | 29 | 1.05 | 45 | 34 | 1691 | 42 | 2970 |
| Dembia | 64 | 127 | 46 | 1.07 | 47 | 30 | 2519 | 15 | 1541 |
| Haramaya | 47 | 140 | 49 | 1.05 | 38 | 28 | 834 | 42 | 1469 |
| Means | 56 | 130 | 36 | 9.9 | 41 | 31 | 1550 | 36 | 2081 |
| CV (%) | 5 | 2 | 15 | 1.1 | 9 | 5 | 15 | 14 | 18 |
| SE± | 0.5 | 0.7 | 0.7 | 0.01 | 0.3 | 0.3 | 37 | 0.7 | 9.9 |
| LSD | 2 | 2 | 3 | 0.1 | 2.2 | 1 | 141 | 3.1 | 56 |

YLD = Grain yield, DF = days to flower, DM = days to mature, PPP= pod per plant, SPP= seed per pod, PHT=plant height, BM= biomass yield, HI= harvest index, HSW= hundred seed weight.

variation in yield potential. The highest mean grain yield was obtained at Debre Zeit (2970 kg/ha) and the lowest was from Haramaya (1469 kg/ha). The possible reason was that late planting was done at Haramaya and due to this moisture stress occurred at vegetative and pod setting stage while relatively sufficient moisture was available at Debre Zeit. Genotypic means across the locations indicated that maximum mean grain yield across all the five locations in one year were obtained from DZ-2012-CK-0013 genotype (2635 kg/ha) and the minimum was from the local variety (1510 kg/ha). Genotype by environment interaction causes differences in yield rank of genotypes in different locations; thus, it becomes important for the chickpea breeders in terms of selection efficiency and genotype suggestions for different locations.

Genotypes showed inconsistent performances across all environments. Genotypes expressed their genetic potential differently in different environments (Table 4). At Akaki, Chefe Donsa, Debre Zeit, Dembia and Haramaya, the top performing genotypes were DZ-2012-CK-0001 (2933 kg/ha), Arerti (3219 kg/ha), Arerti (3560 kg/ha) DZ-2012-CK-0013 (2675 kg/ha) and DZ-2012-CK-0013 (2019 kg/ha), respectively. The genotype DZ-2012-CK-0013 tops performing at Dembia and Haramaya with the average mean yield of 2675 and 2019 kg/ha, respectively. The mean grain yield averaged over environments and genotypes were 2080 kg/ha (Tables 3 and 4). In summary the relative ranking of genotypes at all the five environments were different and CV values of genotype ranged from 2.1 to 18.1% (Table 6).

## Performance of Kabuli-type genotype for yield related traits

From the combined analysis of variance, the mean squares due to genotypes, environments and genotype by environment interaction were highly significant for the traits, days to flowering, days to maturity, plant height, number of pods per plant, hundred seed weight, above ground dry biomass and harvest index. However, there were no-significant effects of all these three source of variation on the number of seeds per pod (Table 3). The separate analysis of variance for all yield related traits, except for number of seed per pod at each location exhibited highly significant (*P≤0.01*) differences among Kabuli-type chickpea genotypes for the days to flowering, days to maturity, number of pods per plant, plant height, hundred seed weight, above ground dry biomass and harvest index at all locations. Similar results were reported by different researchers who worked on chickpea (Singh et al., 1990; Bozoglu and Gulumser, 2000 and Valimohammadi et al., 2007). The responses of genotypes in terms of all yield related traits were different both within and across locations. This indicated that the efficiency of a breeding program aimed at yield improvement is impaired due to genotype by environment interaction, which complicates the process of crop variety development especially when varieties are selected in one environment and used in others (Ahmad et al., 2011).

### *Days to flowering and maturity*

The result reveals significant effects not only for genotypes but also for locations and genotype by environment interaction, variability in experimental material as well as difference in the environmental conditions (Table 3). Early flowering and early maturing genotypes were observed at Haramaya and Debre Zeit (47 and 108 days) and at the same time late flowering and mature genotypes were noted at Dembia and Chefe Donsa (64 and143 days), respectively (Table 5). The probable reason was due to high temperature and early cessation of rain at Haramaya and, relatively long rain season and low temperature at Chefe Donsa. Ejere and

**Table 6.** Mean performance for yield related traits of 17 Kabuli-type chickpea genotypes grown at five environments.

| Genotype | DF | DM | PPP | SPP | PHT | HSW | BM | HI |
|---|---|---|---|---|---|---|---|---|
| DZ-2012-CK-0001 | 58 | 129 | 34 | 1.1 | 39 | 31 | 1775 | 37 |
| DZ-2012-CK-0002 | 57 | 132 | 34 | 1 | 39 | 33 | 1585 | 34 |
| DZ-2012-CK-0003 | 58 | 131 | 35 | 1 | 41 | 35 | 1640 | 32 |
| DZ-2012-CK-0004 | 57 | 129 | 33 | 1 | 38 | 30 | 1487 | 37 |
| DZ-2012-CK-0005 | 59 | 132 | 30 | 1 | 44 | 34 | 1540 | 37 |
| DZ-2012-CK-0006 | 58 | 134 | 28 | 1 | 40 | 37 | 1655 | 31 |
| DZ-2012-CK-0007 | 57 | 131 | 32 | 1 | 46 | 30 | 1585 | 33 |
| DZ-2012-CK-0008 | 60 | 133 | 33 | 1 | 43 | 36 | 1775 | 33 |
| DZ-2012-CK-0009 | 59 | 130 | 33 | 1 | 49 | 31 | 1572 | 36 |
| DZ-2012-CK-0010 | 51 | 131 | 41 | 1 | 43 | 34 | 1420 | 39 |
| DZ-2012-CK-0011 | 57 | 131 | 32 | 1 | 36 | 30 | 1335 | 34 |
| DZ-2012-CK-0012 | 52 | 130 | 34 | 1 | 46 | 31 | 1635 | 35 |
| DZ-2012-CK-0013 | 58 | 131 | 36 | 1 | 41 | 33 | 1810 | 38 |
| Arerti (SC) | 57 | 127 | 43 | 1 | 35 | 24 | 1620 | 41 |
| Ejere(SC) | 49 | 124 | 37 | 1 | 38 | 33 | 1438 | 41 |
| Habru (SC) | 49 | 124 | 39 | 1 | 40 | 30 | 1395 | 43 |
| Dz-10-4(LC) | 57 | 126 | 52 | 1.7 | 39 | 17 | 1078 | 39 |
| Means | 56 | 130 | 36 | 1.1 | 41 | 31 | 1550 | 37 |
| CV (%) | 5 | 2 | 15 | 18.1 | 9 | 5 | 15 | 14 |
| SE± | 0.5 | 1 | 1 | 0.01 | 0.4 | 0.3 | 37 | 0.7 |
| LSD | 2 | 2 | 3 | 0.1 | 2 | 1 | 141 | 3.1 |

DF = Days to flower, DM = days to mature, PPP = pod per plant, SPP = seed per pod, PHT = plant height, BM = biomass yield, HI= harvest index, HSW = hundred seed weight. SC = standard check; LC = local check

Habru were both early flowering and early maturing varieties while the genotypes DZ-2012-CK-0008(60) and DZ-2012-CK-0006 (134) were late flowering and late maturing (Table 6).

### *Number of pods per plant*

Number of pods per plant is an important selection criterion for the development of high yielding genotypes and is strongly influenced by environment in chickpea (Malik et al., 1988). Marked variation was observed in the performance of genotypes over the five locations (Table 3). Number of pods per plant was highest at Haramaya (49) and least at Akaki (25) (Table 5). The genotypes mean values for number of pods per plant varied from 28 for DZ-2012-CK-0006 to 52 DZ-10-4. The highest mean number of pods per plant was recorded for genotypes Dz-10-4 (52) followed by Arerti, DZ-2012-CK-0010 (43), Habru (39) (Table 6). These results are consistent with the findings of Singh and Bains (1984) and Malik et al. (1988). These results indicate variability for number of pods per plant and its sensitiveness to environmental fluctuations.

### *Plant height (cm)*

Significant effects were observed not only for genotypes but also for locations and genotype by environment interaction, reflecting genetic variability in experimental material as well as difference in the environmental conditions (Table 3). Averaged over all genotypes the highest plant height was recorded at Dembia (47 cm) and the shortest was Akaki (36 cm) (Table 5). Plant height was sensitive to environmental fluctuations and it indicated that the relative performance of genotypes was markedly inconsistent over the locations. Averaged over all locations the shortest genotype was Arerti (35 cm) and the longest genotype was DZ-2012-CK-0009 (49 cm) (Table 6). These results are consistent with the findings in chickpea of Malik et al. (1988) who also found high magnitude of genotype by environment interaction.

### *100-grain weight (g)*

Statistically significant variance was observed for genotypes, location and genotype and environment interaction (Table 3). Over all genotypes hundred seed weight was highest for Debre Zeit (34.2 g) and lowest for Haramaya (28.3 g) (Table 5). In addition, the relative performance of genotypes is quite inconsistent across the environments. The genotype with the smallest 100-grain weight was DZ-10-4 (17 g) and the one with the highest was DZ-2012-CK-0006 (37 g) (Table 6). The significant pooled deviation for 100-grain weight suggested that

these genotypes differ considerably with respect to their suitability for this character. The present results are in agreement with the findings of Singh and Singh (1974) and Sanghi and Kandakar (2001).

### *Above-ground dry biomass*

Statistically highly significant variance was observed for genotypes, locations and genotype and locations interaction (Table 3). Averaged across all genotypes above ground dry biomass was highest for Dembia (2519 g) and lowest for Haramaya (834 g) (Table 5).

### *Harvest index*

Statistically highly significant variance was observed for genotypes, locations and genotype and locations interaction (Table 3). Averaged over all genotypes, harvest index was highest for Akaki (47.9%) and least for Dembia (15.1%) (Table 5). Harvest index ranged from (30.7%) for DZ-2012-CK-0006 to (43.7%) for Habru (Table 6). The presence of genetic variation on agronomic traits of Kabuli-type chickpea was similarly reported by Singh et al. (1990) and Bozoglu and Gulumser (2000).

## Stability analysis

### *Wricke's ecovalence analysis*

Wricke's ecovalence (*Wi*) was calculated for each of the 17 Kabuli-type chickpea genotypes evaluated at five diverse locations for one year in the major chickpea growing regions of Ethiopia (Table 7). The genotypes with the lowest ecovalence contributed the least to the genotype by environment interaction and are therefore more stable. Accordingly, DZ-2012-CK-0006, DZ-2012-CK-0011, DZ-2012-CK-0005, DZ-2012-CK-0002, DZ-2012-CK-0009, Ejere and DZ-2012-CK-0007 were the most stable genotypes that for grain yield ranked 15th, 16th, 5th, 13th, 10th, 11th and 14th, respectively. Although, the most unstable genotypes were Arerti, DZ-10-4, DZ-2012-CK-0004, DZ-2012-CK-0013, DZ-2012-CK-0010, DZ-2012-CK-0001, DZ-2012-CK-0012, DZ-2012-CK-0003; that for grain yield ranked 2nd, 17th, 9th, 1st, 8th, 4th, 12th and 7th, respectively. The results indicate that high yielders have high ecovalence and *vice versa*. As a result, genotype recommendation for general adaptability would be difficult. According to Asrat et al. (2008), genotypes with high ecovalence mean and large estimated values are suitable for high input environments.

**Table 7.** Wrickes ecovalence value for 17 Kabuli-type chickpea genotypes at five environments.

| Genotype | Wi | Rank | Mean yield | Rank |
|---|---|---|---|---|
| DZ-2012-CK-0001 | 35315 | 6 | 2304 | 4 |
| DZ-2012-CK-0002 | 5784 | 14 | 1968 | 13 |
| DZ-2012-CK-0003 | 27560 | 8 | 2128 | 7 |
| DZ-2012-CK-0004 | 46768 | 3 | 2042 | 9 |
| DZ-2012-CK-0005 | 5005 | 15 | 2210 | 5 |
| DZ-2012-CK-0006 | 3516 | 17 | 1774 | 15 |
| DZ-2012-CK-0007 | 12104 | 11 | 1962 | 14 |
| DZ-2012-CK-0008 | 19016 | 9 | 2135 | 6 |
| DZ-2012-CK-0009 | 6341 | 13 | 2042 | 10 |
| DZ-2012-CK-0010 | 36075 | 5 | 2090 | 8 |
| DZ-2012-CK-0011 | 4269 | 16 | 1718 | 16 |
| DZ-2012-CK-0012 | 34299 | 7 | 2023 | 12 |
| DZ-2012-CK-0013 | 40149 | 4 | 2635 | 1 |
| Arerti (SC) | 75764 | 1 | 2412 | 2 |
| Ejere (SC) | 10587 | 12 | 2025 | 11 |
| Habru (SC) | 15586 | 10 | 2393 | 3 |
| Dz-10-4 (LC) | 72254 | 2 | 1510 | 17 |

SC = Standard check; LC = Local check

**Table 8.** Sum of square and mean sum of squares from the analysis of variance for linear regressions of Kabuli-type chickpea genotypes means on environmental index according to Eberhart and Russell's joint regression model (1966).

| Source of variation | Df | SS | MS |
|---|---|---|---|
| Total | 339 | 2292863 | |
| Genotype | 16 | 338251 | 21141** |
| Env. + in Gen + Env. | 68 | 1954612 | 28744 |
| Env. in linear | 1 | 1504219 | 1504219** |
| Gen x Env. (linear) | 16 | 77859 | 4866 |
| Pooled deviation | 51 | 372533 | 7305** |
| Residual | 255 | 540506 | 2119 |

**Significant at P≤0.01; Grand mean = 499.365, R-squared = 0.8094, C.V. = 18.44%.

## Eberhart and Russell's joint regression stability analysis

The mean square for genotype by environment significant was ($p \leq 0.01$) for grain yield (Table 3). This permitted the partitioning of genotype by environment effects in environment linear, G x E (linear) interaction effects (sum squares due to regression (bi) and unexplained deviation from linear regression (pooled deviation mean squares ($S^2$di). The genotype by environment (linear) interaction was not significant indicating that the stability parameter 'bi' estimated by linear response to change in environment was the same for all genotypes or genotypes have the same slope (Table 8). Similar results were obtained in bean genotypes tested (Firew, 2003; Setegn and Habtu, 2003) in different part of Ethiopia and in Brazil (Ferreira et al., 2006). Our results reveal that the

**Table 9.** Mean yield, regression coefficients (*b*), coefficients of determination and deviation from regression ($S^2di$) of Kabuli genotype.

| Genotype | $b_i$ | $r_i^2$ | $S^2d_i$ | Mean yield | Rank |
|---|---|---|---|---|---|
| DZ-2012-CK-0001 | 1.68 | 1.01 | 9329 | 2304 | 4 |
| DZ-2012-CK-0002 | 1.43 | 0.99 | 807 | 1968 | 13 |
| DZ-2012-CK-0003 | 1.68 | 1.00 | 1307 | 2128 | 7 |
| DZ-2012-CK-0004 | 1.53 | 1.01 | 6940 | 2042 | 9 |
| DZ-2012-CK-0005 | 1.68 | 0.99 | 761 | 2210 | 5 |
| DZ-2012-CK-0006 | 1.36 | 0.99 | 1043 | 1774 | 15 |
| DZ-2012-CK-0007 | 1.52 | 1.00 | 0.002 | 1962 | 14 |
| DZ-2012-CK-0008 | 1.78 | 1.00 | 4139 | 2135 | 6 |
| DZ-2012-CK-0009 | 1.49 | 1.00 | 260 | 2042 | 10 |
| DZ-2012-CK-0010 | 1.82 | 1.01 | 9665 | 2090 | 8 |
| DZ-2012-CK-0011 | 1.30 | 0.99 | 742 | 1718 | 16 |
| DZ-2012-CK-0012 | 1.80 | 1.01 | 9112 | 2023 | 12 |
| DZ-2012-CK-0013 | 1.82 | 1.00 | 4351 | 2635 | 1 |
| Arerti (SC) | 1.96 | 1.03 | 22135 | 2412 | 2 |
| Ejere (SC) | 1.56 | 1.00 | 1196 | 2025 | 11 |
| Habru (SC) | 1.77 | 1.00 | 2230 | 2393 | 3 |
| Dz-10-4 (LC) | 1.43 | 1.03 | 21350 | 1510 | 17 |

bi = Regression coefficients, $r^2i$. coefficients of determination, $S^2di$. deviation from regression. SC = Standard check; LC = Local check.

genotype by environment interaction was not a linear function of environment indices. The variation among the genotypes and for genotype by environment interaction were significant effects which means that genotypes exhibited different performances in different environments which is due to their different genetic makeup or the variation due to the environments or both. The mean sums of squares due to pooled deviation from regression were significant ($p \leq 0.01$) for grain yield indicating the importance of non linear genotype by environment. The most stable genotype with the lowest $S^2di$ values were DZ-2012-CK-0007($s^2di=0.002$), DZ-2012-CK-0009, DZ-2012-CK-0011, DZ-2012-CK-0005 and DZ-10-2012-CK-0002 in decreasing order. The most unstable genotype with the highest $S^2di$ values were Arertie, DZ-10-4, DZ-2012-CK-0010, DZ-2012-CK-0001, DZ-2012-CK-0012, DZ-2012-CK-0004, DZ-2012-CK-0013 and DZ-2012-CK-0008 which ranked 1st, 2nd, 3rd, 4th, 5th, 6th, 7th and 8th, respectively. Therefore, these genotypes were best fit for specific adaptation in favorable environments where there were high levels inputs. If the mean yield, regression coefficient value (*bi*) and the deviation from the regression ($S^2di$) are considered together simultaneously, there was no stable genotype. All genotypes had regression coefficients (bi) greater than one (that is, below average stability and significant deviation from regression). Therefore, these genotypes were specifically adapted to favorable environments (Table 9).

## AMMI analysis of 17 Kabuli-type chickpea genotypes tested at five environments

The AMMI analysis of variance of grain yield of 17 Kabuli-type chickpea genotypes tested in five environments is presented on Table 7. The analysis revealed that Kabuli-type chickpea genotypes were significantly ($P \leq 0.01$) affected by environments (E), genotypes (G) and genotype by environment interaction. The main effects of E and G accounted for 53.1 and 11.9%, respectively, and G X E interaction accounted for 15.9% of the total variation of Genotype by environment data for grain yield (Table 10). The first two principal components (PC1 and PC2), which were used to create a two-dimensional biplot, explained 52.5 and 21.95% of AMMI sum of squares, respectively. According to the AMMI model, the genotypes which are characterized by means greater than grand mean and the IPCA score nearly zero are considered as generally adaptable to all environment (Ezatollah et al., 2013). However, the genotype with high mean performance and with large value of IPCA score are consider as having specific adaptability to the environments. The large sum of squares for environments showed that the environments were diverse, with large differences among environmental means causing most of the variation in grain yield. This is in synchronization with the findings of Singh et al. (1990), Yan (2002) and Yan and Tinker (2006) in chickpea

**Table 10.** Additive Main effects and Multiplicative interaction (AMMI) analysis of variance for grain yield (kg/ha) of the 17 Kabuli-type genotypes tested across five locations.

| Source | Df | Sum of squares | Mean squares | % Explained |
|---|---|---|---|---|
| Total | 339 | 11333475 | 33432 | |
| Environment (L) | 4 | 6016879 | 1504220** | 53.1 |
| Genotype (G) | 16 | 1353003 | 84563** | 11.9 |
| G × L | 64 | 1801570 | 28150** | 15.9 |
| IPC1 | 19 | 945954 | 49787** | 52.5 |
| IPC2 | 17 | 395464 | 23263** | 21.9 |
| IPCA3 | 15 | 237463 | 15831* | 13.2 |
| Residuals | 13 | 222688 | 17130 | |

*,**Significant at P≤0.05 and P≤0.01, respectively.

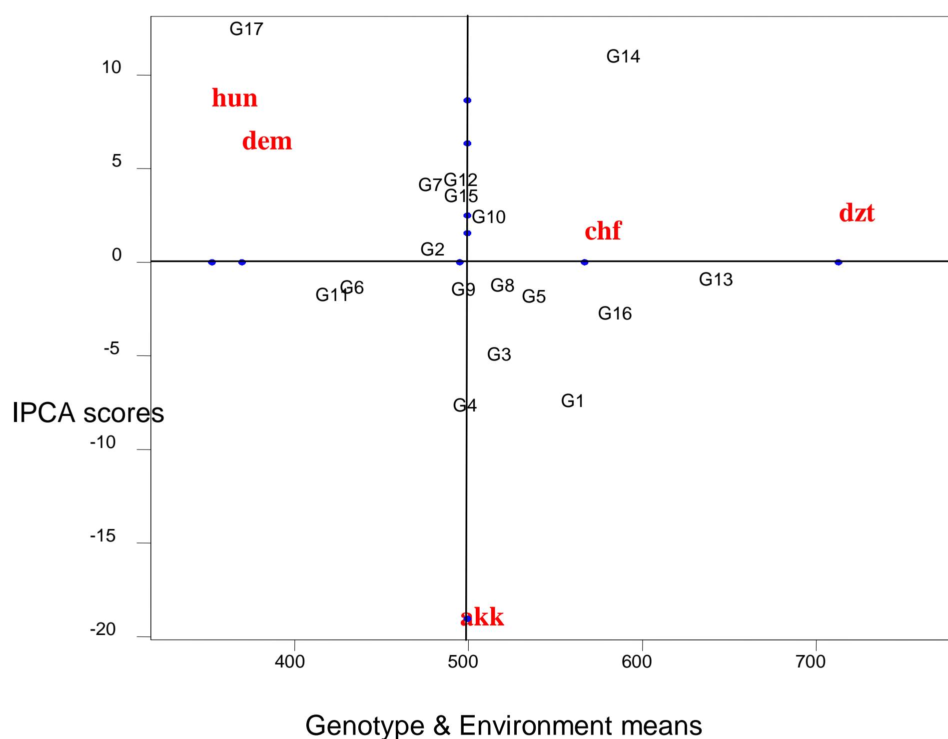


**Figure 1.** AMMI biplot analysis of IPCA scores genotype and environment means for Kabuli-type genotypes.

production. This result also indicates the considerable influence of environments on the yield performance of Kabuli-type chickpea genotypes in Ethiopia. The magnitude of the genotype by environment sum of squares was more than two times that for genotypes, indicating that there were considerable differential genotype responses across environments.

The AMMI I, biplot for grain yield of the 17 Kabuli-type chickpea genotypes at five environmental conditions is shown in Figure 1. The main effects (genotypes and environments) accounted for 65.02% of the total variation and IPCA 1 accounted for 52.5% of the total variation due to genotype by environment interaction alone. Environments showed high variation in both main effects and interactions (IPCA1) (Figure 1). Chefe Donsa and Debre Zeit are the most favorable environments; Haramaya and Dembia are the least favorable environments, while Akaki is the average environment. Environments are classified into three main groups based on their IPCA 1 scores Haramaya and Dembia are in quadrant I and have got large positive IPCA1 scores, which interact positively with genotypes that have positive

IPCA1 scores and negatively with those genotypes having negative IPCA1 scores. Chefe Donsa and Debre Zeit in quadrants II and have got small positive IPCA1 scores, which interact positively with genotypes that have positive IPCA1 scores and negatively with those genotypes having negative IPCA1 scores; Akaki in quadrant III and has got large negative IPCA1 scores which interact negatively with genotypes having negative IPCA1 scores and positively with genotypes having positive IPCA1 scores; and Akaki is in quadrant III and has got large negative IPCA1 scores which interacts negatively with genotypes that have negative IPCA1 scores and positively with those genotypes having positive IPCA1 scores (Table 10). The environments can be sub-grouped according to their average yield over the genotypes. According to environmental IPCA1 scores, Akaki, Chefe Donsa and Debre Zeit were more stable and had lower genotype by environment interaction, and had high yield performance. On the other hand, the highest IPCA1 scores belonged to Haramaya and Dembia, but they had low yield performance. According to IPCA1, environment Debre Zeit was an ideal environment for selecting genotypes with specific adaptation to high input conditions.

The IPCA 1 and IPCA 2 components were significant (*P≤0.01*) and accounted for 52.5 and 21.95% of the total G X E interaction sum of squares, respectively. The two of them explained more than 74.45% of the total G X E interaction variation (Table 10). This indicates that the AMMI biplot model is the best fit for this data set, which is in agreement with other studies (Zobel et al., 1988; Yan and Hunt, 1988). In Figure 1, the genotypes and locations that are located far away from the origin are more responsive. Haramaya, Debre Zeit, Dembia and Chefe Donsa are the most differentiating environments, while Akaki is more responsive environment than the other environments since it is far away from the origin. The genotypes DZ-2012-CK-0003, DZ-2012-CK-0008 and DZ-2012-CK-0013 were the most stable as well as productive. DZ-2012-CK-0005, Habru and DZ-2012-CK-0009 were stable with intermediate productivity. DZ-10-4 and Arerti are less responsive. Genotypes and environments that fall into the same sector interact positively; negatively if they fall into opposite sectors (Osiru et al., 2009). A genotype showing high positive interaction in an environment obviously has the ability to exploit the agro-ecological or agro-management conditions of the specific environment. If they fall into adjacent sectors, interaction is somewhat more complex. In this case, the best genotype with respect to Akaki site are DZ-2012-CK-0003, DZ-2012-CK-0005, DZ-2012-CK-0013, DZ-2012-CK-0001, DZ-2012-CK-0008 and Habru with respect to Chefe Donsa and Debre Zeit they were DZ-2012-CK-0010 and Arerti and DZ-10-4, DZ-2012-CK-0002, DZ-2012-CK-0012 and Ejere were the best genotype for environments of Dembia and Haramaya. DZ-2012-CK-0001=G1, DZ-2012-CK-0002=G2, DZ-2012-CK-0003=G3, DZ-2012-CK-0004=G4, DZ-2012-CK-0005=G5, DZ-2012-CK-0006=G6, DZ-2012-CK-0007=G7, DZ-2012-CK-0008=G8, DZ-2012-CK-0009=G9, DZ-2012-CK-0010=G10, DZ-2012-CK-0011=G11, DZ-2012-CK-0012=G12, DZ-2012-CK-0013=G13, Arerti=G14, Ejere=G15, Habru=16 and Dz-10-4=G17, Chf= Chefe Donsa, Akk=Akaki, Dzt=Debre Zeit=Dem=Dembia and Hun=Haramaya.

### AMMI stability value

According to the ASV ranking, the most stable genotypes were DZ-2012-CK-0002, DZ-2012-CK-0006 and DZ-2012-CK-0009. DZ-2012-CK-0013 and Arerti which were the first and second highest yielders based on the mean yield values (Table 11). However, DZ-2012-CK-0013 which gave the highest mean yield, ranked 12$^{th}$ for the ASV. The most unstable genotypes according to the ASV were DZ-10-4 and DZ-2012-CK-0004 (Table 11).

### Cluster analysis of genotypes and environments

Cluster analysis was performed to study the patterns of groupings of genotypes and environments. The dendrograms (Figures 2 and 3) were generated from SAS clustering method of genotypes and environments based on Euclidean distances using AMMI adjusted mean yields of genotypes and environments, respectively. Clustering of genotypes at a cut-off value of zero produced five clusters. Cluster one consisted of 11 genotypes (DZ-2012-CK-0001, DZ-2012-CK-0002, DZ-2012-CK-0003, DZ-2012-CK-0004, DZ-2012-CK-0005, DZ-2012-CK-0007, DZ-2012-CK-0008, DZ-2012-CK-0009, DZ-2012-CK-0010, DZ-2012-CK-0012 and Ejeri). Cluster two consisted of two genotypes (Arerti and Habru). The third cluster also consisted of two genotypes (DZ-2012-CK-0006 and DZ-2012-CK-0011). The fourth group consisted of only one genotype (DZ-2012-CK-0013), and this genotype is the highest yielder of all the 17 Kabuli-type chickpea genotype. The last group included only one genotype local variety which was the lowest yielder of all the 17 genotypes. Cluster analysis of environments at cut-off point 1.0 produced three clusters, two of which consisted of two environments each and the third cluster consisted only one environment. Chefe Donsa and Debre Zeit were in the first group. The second cluster included only Dembia and the third cluster consisted of Akaki and Haramaya.

### Conflict of interests

The authors did not declare any conflict of interest.

### ACKNOWLEDGMENTS

We would like to express our appreciation to TL-I Project

**Table 11.** Yield and parametric stability statistics for grain yield on 17 Kabuli genotypes grown in five environments.

| Genotype | IPCA1 | IPCA2 | ASV | Rank | Yield |
|---|---|---|---|---|---|
| DZ-2012-CK-0001 | -7.83 | 2.64 | 11.81 | 14 | 2304 |
| DZ-2012-CK-0002 | 0.23 | 0.32 | 0.48 | 1 | 1968 |
| DZ-2012-CK-0003 | -5.34 | 5.17 | 9.74 | 13 | 2128 |
| DZ-2012-CK-0004 | -8.08 | 2.15 | 12.67 | 15 | 2042 |
| DZ-2012-CK-0005 | -2.27 | -1.39 | 3.77 | 4 | 2210 |
| DZ-2012-CK-0006 | -1.76 | -2.18 | 3.49 | 2 | 1774 |
| DZ-2012-CK-0007 | 3.71 | 0.07 | 5.74 | 7 | 1962 |
| DZ-2012-CK-0008 | -1.69 | -5.32 | 5.93 | 9 | 2135 |
| DZ-2012-CK-0009 | -1.89 | -2.01 | 3.55 | 3 | 2042 |
| DZ-2012-CK-0010 | 1.98 | -7.25 | 7.87 | 11 | 2090 |
| DZ-2012-CK-0011 | -2.18 | 1.85 | 3.84 | 5 | 1717 |
| DZ-2012-CK-0012 | 3.97 | 1.23 | 4.16 | 6 | 2023 |
| DZ-2012-CK0013 | -1.35 | -9.45 | 9.68 | 12 | 2635 |
| Arerti (SC) | 10.55 | 6.79 | 17.67 | 16 | 2412 |
| Ejere (SC) | 3.11 | 3.16 | 5.75 | 8 | 2025 |
| Habru (SC) | -3.18 | 5.66 | 7.49 | 10 | 2393 |
| Dz-10-4 (LC) | 12.04 | -1.43 | 18.67 | 17 | 1510 |

Wi2, Wricke´s ecovalence; $S^2di$, deviation from regression; bi, regression coefficient; $r^2i$, coefficient of determination; IPCA1 and IPCA2, interaction principal components axes 1 and 2, respectively; ASV, AMMI stability value; SC, standard check; LC, local check.

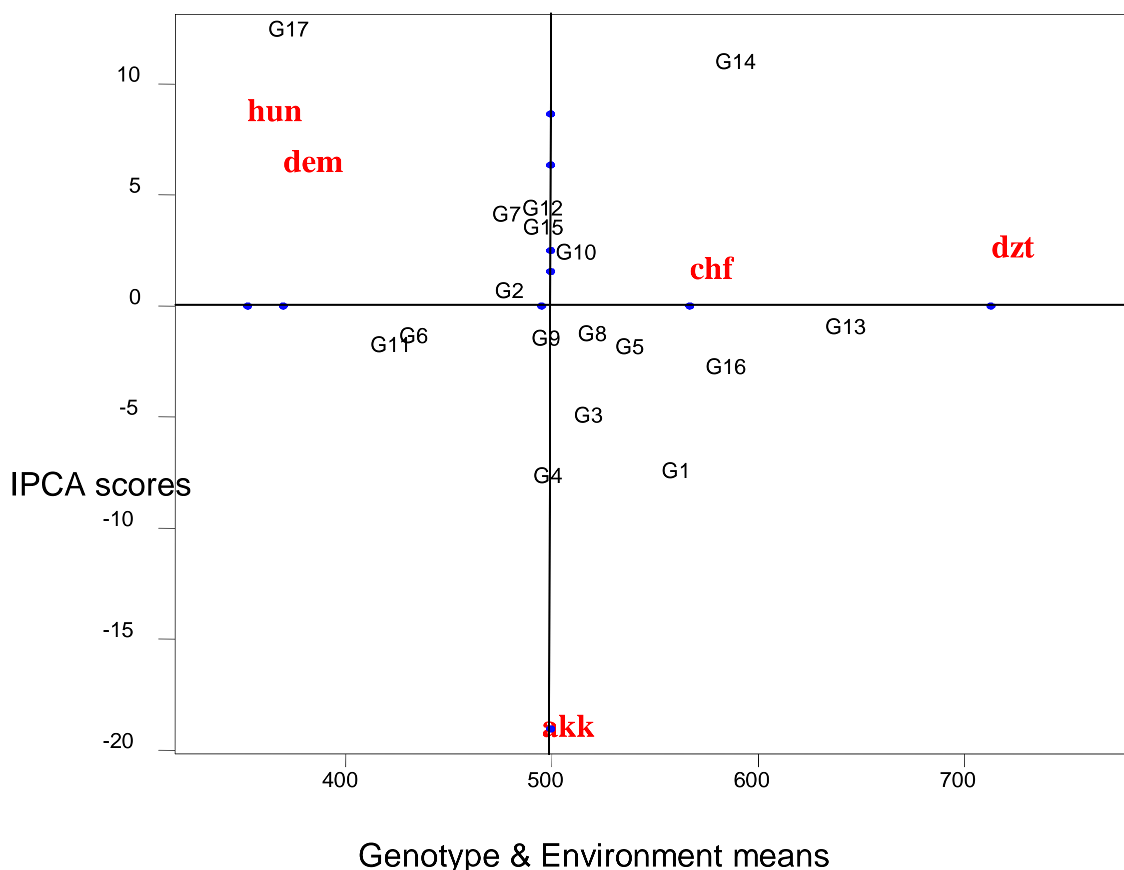


**Figure 1.** AMMI biplot analysis of IPCA scores genotype and environment means for Kabuli-type genotypes.

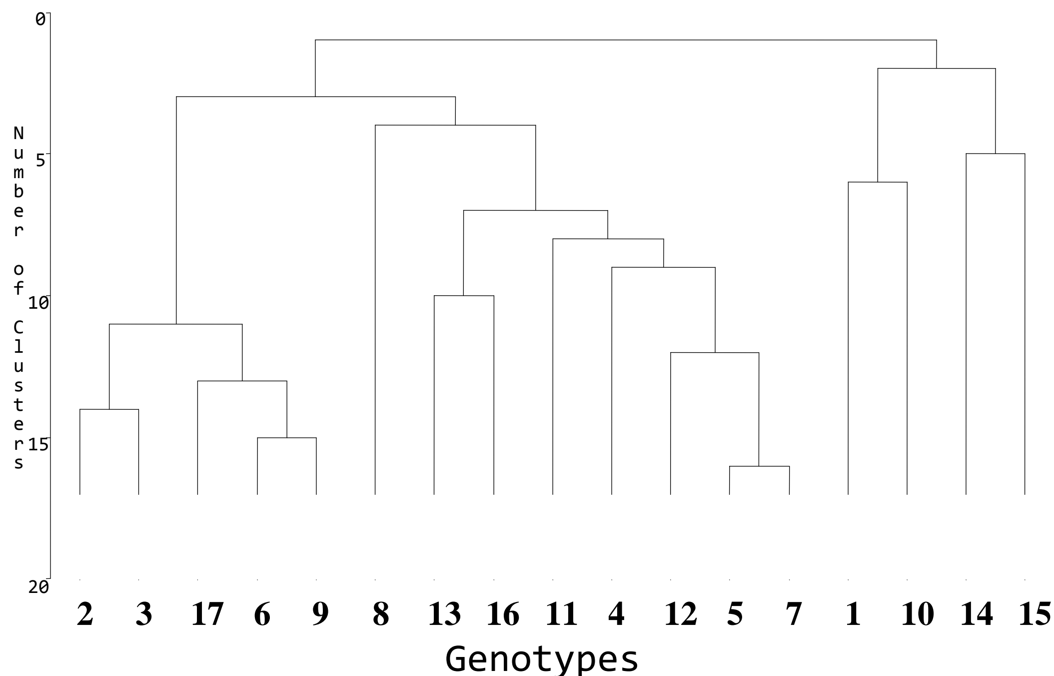


**Figure 2.** Dendrogram illustrating cluster analysis of Kabuli-type chickpea genotypes. DZ-2012-CK-0001=1, DZ-2012-CK-0002=2, DZ-2012-CK-0003=3 DZ-2012-CK-0004=4, DZ-2012-CK-0005=5, DZ-2012-CK-0006=6, DZ-2012-CK-0007=7, DZ-2012-CK-0008=8, DZ-2012-CK-0009=9, DZ-2012-CK-0010=10, DZ-2012-CK-0011=11, DZ-2012-CK-0012=12, DZ-2012-CK-0013=13, Arerti=14, Ejere=15, Habru=16 and Local variety=17.

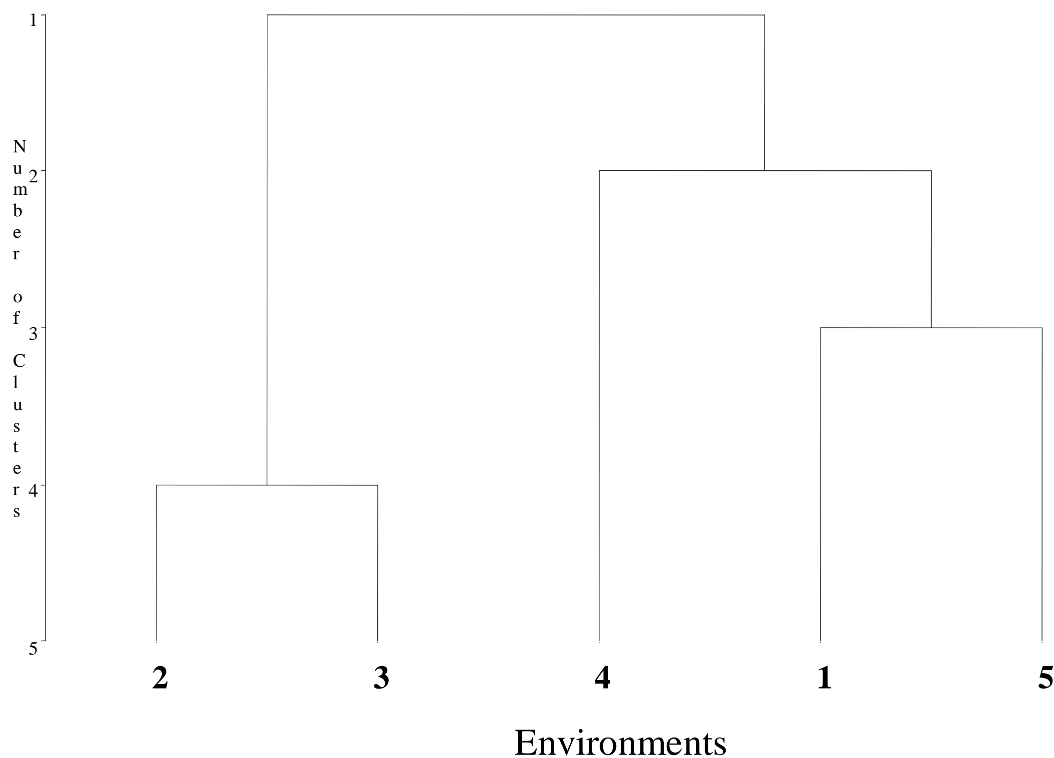


**Figure 3.** Dendrogram illustrating the clustering of five environments for Kabuli-type chickpea genotypes. Akaki=1, Chefe Donsa=2, Debre Zeit=3, Dembia=4 and Haramaya=5.

for its financial support. We are also indebted for the assistances provided by the research and technical staff of the highland Pulse Improvement Program of Debre Zeit Agricultural Research Center (DZARC) and Gonder Agricultural Research Center (GARC).

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Vol. 14(18), pp. 1576-1583, 6 May, 2015
DOI: 10.5897/AJB201514443
Article Number: C69E66352713
ISSN 1684-5315
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African Journal of Biotechnology

*Full Length Research Paper*

# Effects of the humic acid extracted from vermicompost on the germination and initial growth of *Brachiaria brizantha* cv. MG5

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Received 20 January, 2015; Accepted 7 April, 2015

**The biological effects of humic substances on vegetables depend on the source of extraction and the concentration used, on the vegetable species and on the age of the plant. This study aimed to evaluate the effect of different humic acid (HA) doses extracted from vermicompost on the germination and initial growth of *Brachiaria brizantha* cv. MG5. To that end, germination tests were conducted in germination agents, as well as emergence test in greenhouse and seedlings growth test for those agents. For all these tests, five doses of HA [0.0; 1.0; 2.0; 4.0 e 8.0 mM C.L$^{-1}$] were used, with and without reapplying HA. The following characteristics were analyzed: percentage of germination, percentage of normal seedlings in the first counting of the germination test, percentage of abnormal seedlings, percentage of non-germinated seedlings, percentage of emergence, emergence speed rates, fresh and dry matter of the upper and roots part, length of the upper and roots part and number of lateral roots. Positive effect of HA reapplication in the percentage of abnormal seedlings was observed. With increasing doses of HA, there was a reduction in the percentage of germination and normal seedlings in the first count. The application of HA promoted increase in shoot length and number of lateral root. There was no significant difference in the length of the root. Humus acid affects positively the initial growth of *B. brizantha* cv. MG5, with the best stimulation being observed when the 2.0 mM C.L$^{-1}$ dose was applied.**

**Key words:** Forage, *Urochloa* sp., vigor.

## INTRODUCTION

Organic matter for soils, waters and sediments have humic substances as their main component. They influence chemical, physical and biological properties and directly affect the growth and metabolism of plants, especially the root system (Nardi et al., 2002). Vermicomposting is the post-thermophilic biodegradation of organic material through the interaction between earthworms and microorganisms (Edwards et al., 2010). The mature vermicompost is significantly enriched in humic acids, which have a well- acknowledged capability

to induce plant development, especially for root systems (Canellas et al., 2010; Muscolo et al., 2013). The most studied physiological effects of the humic substances regard the promotion of root growth (Façanha et al., 2002; Rodda et al., 2006a; Zandonadi et al., 2007). The great majority of the biostimulating effects of humus acid (HA) has been credited to its similar activities to the auxins (Chen and Aviad, 1990; Canellas et al., 2002; Façanha et al., 2002), considering that these effects on the vegetable growth depend on the type of source from where the humic substances are isolated, on the type and age of the plants and on the concentration used for the essays (Kononova, 1982; Santos and Camargo, 1999; Muscolo et al., 2013; Martinez-Balmori et al., 2014).

*Brachiaria* seeds have difficulty to germinate in the laboratory and in the field, and the main factor contributing to this is the occurrence of natural or innate dormancy, presenting among other factors, heterogeneity of maturation (Lago and Martins, 1998). In forage species, the formation of a dense stand at sowing is fundamental to the pasture productivity (Sulc, 1998). For this to happen, there needs to be effected quickly and evenly seedling emergence, allowing the formation of a closed canopy early in the growing season, suppressing weeds and maximized light interception. A suggested hypothesis is that the positive biological effects of HS in the plant can be due to an auxin activity (Nardi et al., 2002; Piccolo et al., 1992). To this effect, auxin promotes acid growth of plants in which the cell elongation and the consequent increase in length of the plants is a function of cell turgor. This increased growth promoted by humic acid can be of great importance in the early growth of brachiaria seedlings, promoting rapid establishment of forage resulting in higher quality pastures. Given in the above, testing the hypothesis that humic acids affect the growth of *Brachiaria brizantha* cv. MG5 plants, related to the used dose and to the reapplication of the solution, we aimed to evaluate the effect of different doses of humus acid extracted from vermicompost on the germination and initial growth of *B. brizantha* cv. MG5.

## MATERIALS AND METHODS

Two experiments were separately performed in germination chamber Biochemical Oxygen Demand (BOD) type and in a greenhouse to evaluate the effect of HA on the initial growth of branquiaria seedlings. All tests were performed with commercial seeds of *B. brizantha* cv. MG5 ventilated with a DeLeo blower, with a 4.8 cm of aperture aiming to remove impurities and empty seeds, obtaining the pure seed fraction to conduct the tests. The humus acid used was extracted from a vermicompost obtained through the personal collection existent in the Laboratory of Environmental Microbiology and Biotechnology of the University of Vila Velha, Brazil. A vermicompost was obtained from mixture of plant residues from *Panicum maximun* Jacq., and cattle manure 5:1 (v/v). The organic residues were mixed and earthworms were added at a ratio of 5 kg earthworms (*Eisenia foetida*) per $m^3$ of organic residue. A bed of worms and organic residues was first prepared in a container and additional layers of organic residues were periodically placed over the pile as a function of temperature until the pile reached 50 cm. At the end of the transformation process (three months after addition of the last organic residues), worms were removed into a pile of fresh organic residue (plant + cattle manure) placed in a corner of the container. The organic matter composition of the resulting vermicompost was: pH 7.8, 46.5 g $kg^{-1}$ total organic carbon and 17.3 g $kg^{-1}$ HA carbon. HA were isolated from vermicompost and purified as reported elsewhere (Canellas et al., 2002). The HA were suspended in distilled water and titrated to pH 7.0 by automatic titrator (VIT 909 Videotitrator, Copenhagen) with a 0.1 KOH solution under $N_2$. The resulting potassium-humates were then passed through a 0.45 µm Millipore filter and freeze-dried (Canellas et al., 2010). For the usage in the experiments, lyophilized HA was solubilized with a 0.1 mM NaOH solution and it was diluted using a 2 mM $CaCl_2$ solution. The pH solution was set to be between 5.8 and 6.0 and so the dilution of the stock solution with 2 mM $CaCl_2$ solution was proceeded to obtain doses of 0.0, 1.0, 2.0, 4.0 and 8.0 mM C.$L^{-1}$ of HA based on Canellas et al. (2010), which were used in all experiments.

On the first experiment, tests of germination in BOD and emergence tests in greenhouse were conducted. The HA doses were directly applied on the substrate, with and without reapplication of HA. In the treatment with reapplication of HA, the substrate was dampened again on the seventh and fourteenth days with HA, and on the other days it was dampened with water for another time when it was necessary. Regarding the treatment without a reapplication of HA, the substrate was dampened again with water only during all the test conduction.

### Germination test

This test was conducted according to the rules for the analysis of seeds (Brasil, 2009), following a totally randomized outlining with four repetitions of 50 seeds each, by treatment. The seeds were uniformly distributed in gerbox for germination; measuring 11 × 11 × 3.5 cm, over two germitest sheets of paper dampened with 5 ml of AH solution. The gerbox containing the seeds were taken to BOD-type germination agents with a photoperiod of 16/8 h (E/L) and using an alternate temperature of 20 and 35°C, considering 16 h for the lowest temperature and 8 h for the highest one. The treatment with reapplication of HA was dampened once again with 1.0 ml of HA solution in the seventh and fourteenth days, using the same concentrations applied initially. The counting was carried out in the seventh and twenty-first day after the beginning of the test to evaluate normal, abnormal and non-germinated seedlings.

### Emergence test in greenhouse

The emergence test of seedlings in greenhouse followed the outlining in randomized blocks, using four blocks, and it was established in plastic trays perforated at the bottom, using as a

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**Abbreviation: HA,** Humic acid.

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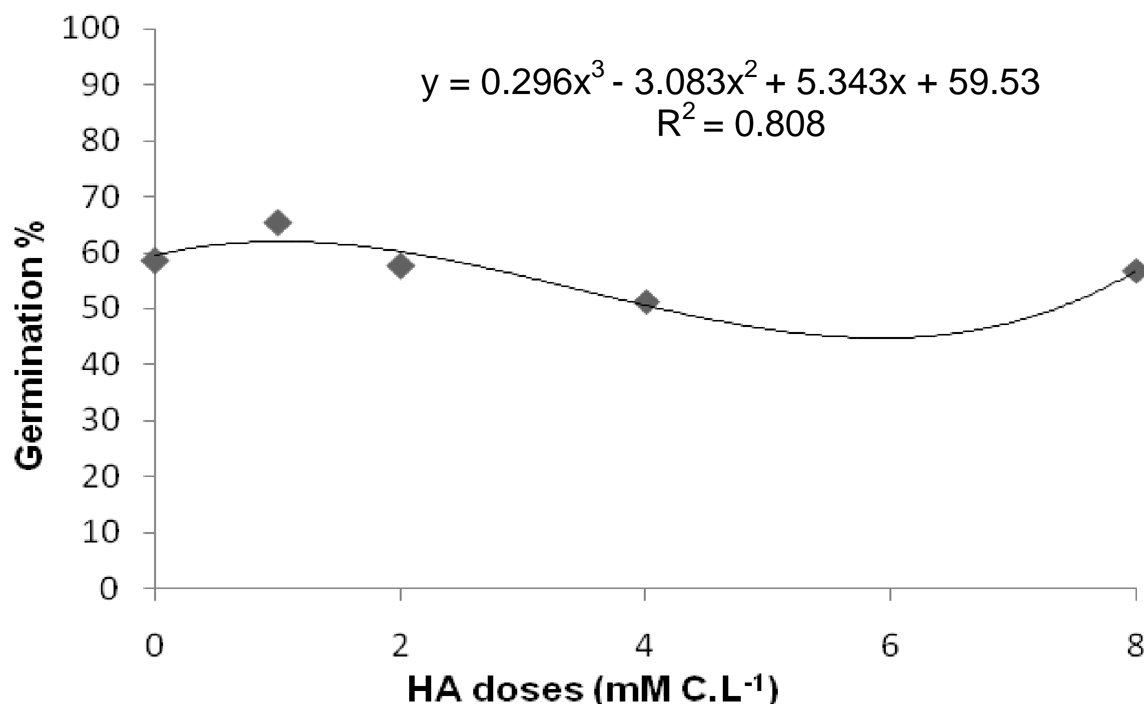


**Figure 1.** Germination percentage of braquiaria seedlings treated with different HA doses.

substrate a soil classified as yellow dystrophic Latosol, mixed with sand in the proportion of 2 x 1 (2 parts of soil for 1 part of sand). The substrate was dampened with 500 ml of HA solution, a volume which is enough to reach the field capacity. Therefore, 50 seeds were seeded by tray with a depth of 0.5 cm. The treatment with reapplied HA was dampened again with 100 ml of HA solution in the seventh day, using the same concentrations applied initially. Daily countings were performed regarding the number of plants emerged to calculate the emergence speed rate. The evaluation of the total of seedlings emerged was performed in the 14th day after the sowing, when the plants were carefully taken from the trays and cut in a way that the upper part of the roots could be separated from the rest. They both were packed in paper bags and weighted in a precision balance to determinate the fresh matter, followed by the determination of the dry matter according to the methodology described by Silva and Queiroz (2006).

The second experiment conducted in a germination agent aimed to evaluate the length of the upper part and the root, as well as the emission of secondary roots of braquiaria seedlings treated with different HA doses directly applied in the substrate in a single time before the sowing, with a solution volume equivalent to three times the weight of the paper being used. The substrate used was a paper roll with three germitest paper sheets, where 20 seeds of *B. brizantha* cv. MG5 were planted with four repetitions by treatment. The paper rolls were packed in plastic bags, which were arranged in plastic trays. They were taken to BOD, where they were put in a way that the inclination was set to 45° to guarantee the action of gravitropism in the growth of the roots. The test was conducted with a photoperiod of 16/8 h (E/L) and with an alternate temperature of 20 and 35°C, considering 16 h to the lowest temperature and 8 h to the highest one. After the germination, only 10 seedlings by repetition were maintained. The evaluations were performed in the 14th day after the sowing. Measures of the upper part and of the roots of all plants were taken. Then, with the help of a loupe, the number of secondary roots by plant was counted. The results obtained were submitted to the analysis of variance (ANOVA) and to regression analysis with the ASSISTAT software.

## RESULTS AND DISCUSSION

No effect of the reapplication of HA to the variable percentage of germination was verified, where it was observed as a reduction in the germination percentage with the increase in the doses, with the greatest reduction happening when a 4.0 mM $C.L^{-1}$ dose was applied (Figure 1). However, in the highest dose, there was a positive stimulus of HA again in the germination percentage. This behavior may have happened in function of the bioactivity of the HA in brachiaria seeds having been affected by the degree of seeds ripeness, since they have an uneven ripeness inside the panicle (Martins and Silva, 2001). They have, a same lot, seeds with different degrees of maturity, which influences the permeability of the tegument and possibly the sensibility of these seeds to distinct HA doses. The results of this study confirm those by Ayuso et al. (1996), that also observed a reduction in the germination percentage in dampened paper substrate with an HA solution, considering that this effect was mainly observed in seeds with a greater sensibility to negative external factors.

According to Azam and Malik (1983), the best effects of the dampened materials in the germination of seeds happen when they are immersed in the solution before the germination itself and when they are germinated in

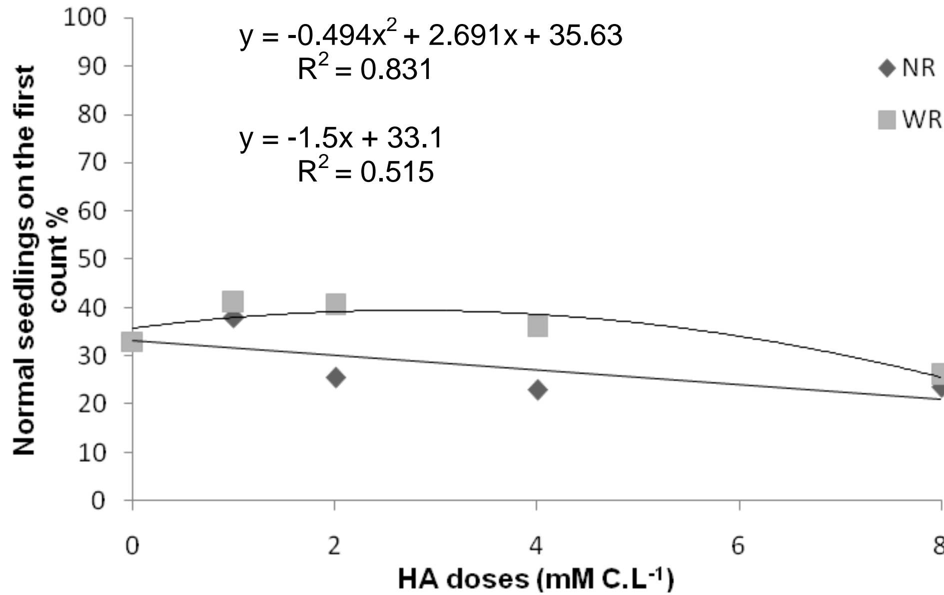


**Figure 2.** Percentage of normal seedlings on the first counting of the test on germination of braquiaria seedlings treated with different HA doses with no reapplication (NR) and with reapplication (WR) of HA.

water. It is better than when they are germinated in HA solutions. It probably happens because the humic substances penetrate the seeds tissues during the immersion (Ayuso et al., 1996). To the normal seedling on the 1st counting, a negative effect was observed when a higher level of HA doses was used, which suggests that there is a decrease on the vigor of the seeds when submitted to higher HA doses (Figure 2). This decrease of vigor indicates a reduction on the germination speed, which may have happened because of the increase on the time of soaking caused by a difference in the osmotic potential in the HA solution. Another factor that may have influenced the response to the HA doses would be the sensibility to external conditions presented by this species of seed, since there are other species with a greater sensibility to negative external factors, such as, the ones suggested by Zucconi et al. (1985) for seeds of watercress and by Ayuso et al. (1996) for seeds of tobacco and watercress. A significant effect on the reapplication of HA in the percentage of normal seedlings was verified (Figure 3). When the seeds were not submitted to reapplication of HA, a linear increase of the percentage of abnormal seedlings was observed with the increase of doses too. However, when HA was reapplied, a contrary effect was demonstrated, because there was a decrease on the percentage of abnormal seedlings increasing the HA doses. The reduction in the percentage of abnormal seedlings in function of the increase of the doses when HA was reapplied may be explained by the fact that the HA solution retards the hydration of the tissue and the gas exchanges, allowing a longer time to repair or reorganize the plasma membranes, also allowing the tissues to be formed in a more organized form, reducing the risks of damage to the embryonic axis (McDonald, 2000; Windauer et al., 2007). This way, the chances of generating abnormal seedlings also decrease. There was no significant difference to the following characteristics: percentage of non-germinated seeds, percentage of emergence and emergence speed rate, in which no effects of none of the doses tested in none of the treatments were observed.

In Table 1, the data of fresh and dry matter in the upper part and in the root, as well as the perceptual variation in relation to the control regarding the function of HA doses and the reapplication or non-reapplication of HA solution are presented. The greatest increments on the fresh matter, as in the upper part (19.48%) as in the root (25.58%), were observed when a 4.0 mM $C.L^{-1}$ was applied with no reapplication of HA. In the treatment with the reapplication of HA, a positive effect of HA was verified, being the 8.0 mM $C.L^{-1}$ the one that presented the best response, increasing the fresh matter of the upper part (5.54%) and of the root (16.56%), and dry part of the upper part (6.77%) and of the root (12.5%) when compared to the control. In general, a greater increment in the fresh matter of the upper and root parts was observed in relation to the dry matter of the upper and root parts, indicating that the stimulus stated in the growth of the plants could be hypothetically attributed to the action of humic acids on the cell elongation by vacuum turgidity (Rayle and Cleland, 1992). Any significant effect of the doses for the root length was identified. In Figure 4, the data of the length of the shoot are presented. Although, regarding the growth of the

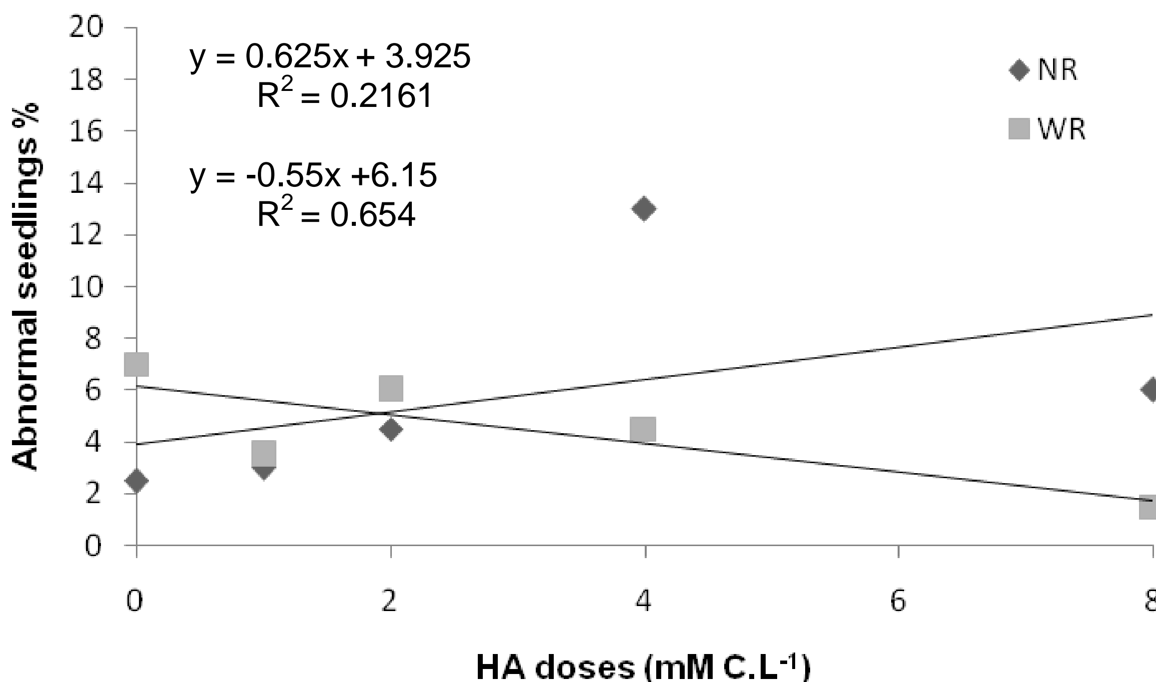


**Figure 3.** Percentage of abnormal seedlings on germination test of braquiaria seeds treated with different HA doses with no reapplication (NR) and with reapplication (WR) of HA.

**Table 1.** Fresh and dry matter in the upper and root part of braquiaria plants in response to different HA doses with or without reapplication of HA.

| HA (mM C.L-1) | Dry matter of shoot (mg/pl) | Fresh matter of the root (mg/pl) | Dry matter of shoot (mg/pl) | Fresh matter of the root (mg/pl) |
|---|---|---|---|---|
| **No reapplication of HA** | | | | |
| 0.0 | 99.33 | 64.37 | 8.06 | 5.56 |
| 1.0 | 104.46 (5.16) | 63.67 (-1.09) | 8.54 (5.95) | 5.48 (-1.44) |
| 2.0 | 102.29 (2.98) | 52.79 (-17.99) | 8.30 (2.98) | 3.56 (-35.97) |
| 4.0 | 118.68 (19.48) | 80.19 (24.58) | 7.86 (-2.48) | 4.97 (-9.17) |
| 8.0 | 92.52 (-6.85) | 66.07 (2.64) | 7.41 (-8.06) | 4.50 (-19.06) |
| **With reapplication of HA** | | | | |
| 0.0 | 104.51 | 62.36 | 8.72 | 4.56 |
| 1.0 | 99.51 (-4.78) | 71.94 (15.36) | 8.56 (-1.83) | 5.00 (9.65) |
| 2.0 | 99.86 (-4.45) | 60.88 (2.37) | 8.23 (-5.62) | 3.90 (-14.47) |
| 4.0 | 90.91 (-13.01) | 53.37 (-14.42) | 8.04 (-7.80) | 3.19 (-30.04) |
| 8.0 | 110.30 (5.54) | 72.69 (16.56) | 9.316.77) | 5.13 (12.5) |

*The values between brackets represent the percentage variation in relation to the control.

upper part, two peaks of HA bioactivity in the seedlings growth could be observed, considering that this happened when 2.0 mM $C.L^{-1}$ and 8.0 mM $C.L^{-1}$ doses were applied.

A dose effect on the number of lateral roots was observed, in which there was an increase in the number of lateral roots with the increase of the doses, reaching a maximum and decreasing again when the highest dose was applied (Figures 5 and 6). The dampened organic matter extracted from the worm composting presents a confirmed hormonal activity (Muscolo et al., 1999; Canellas et al., 2002). The possible presence of growth inducing substances of roots of the auxins type in the worm composting humate promotes the development of

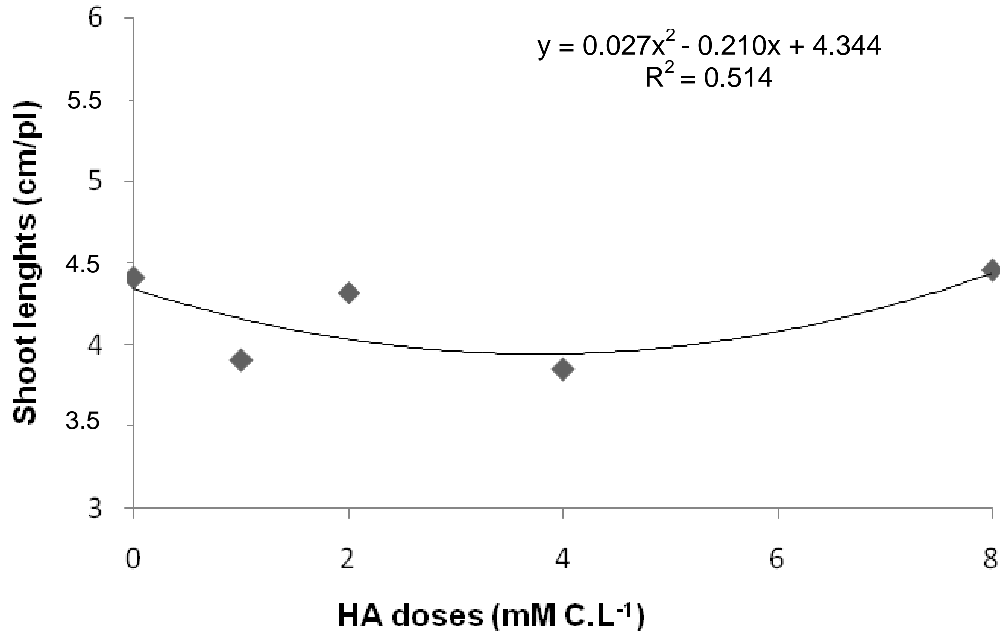


**Figure 4.** Shoot lengths of braquiaria seedlings in response to different HA doses.

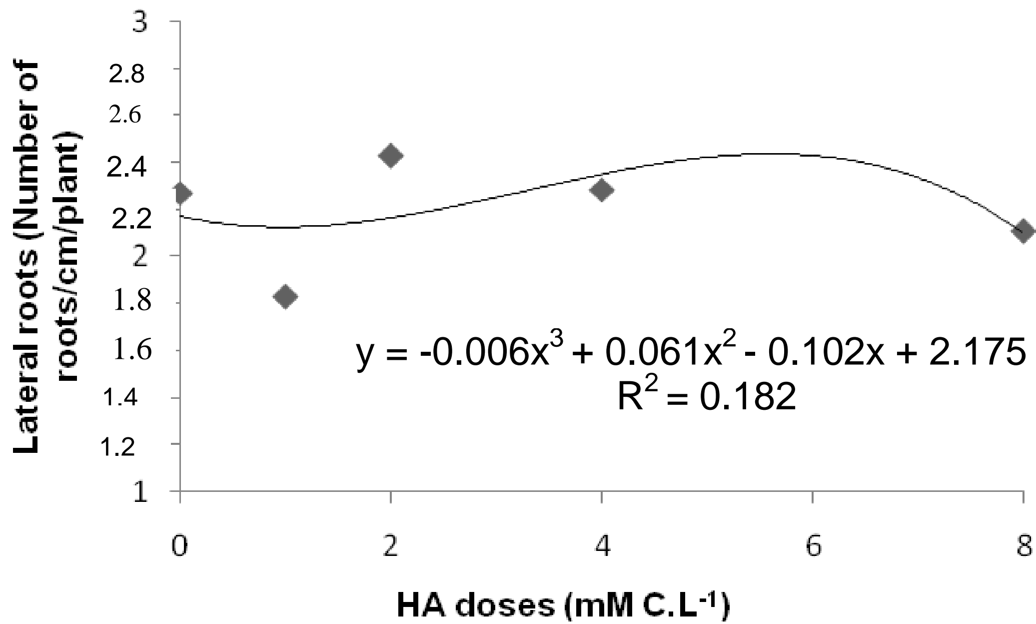


**Figure 5.** Number of lateral roots of braquiaria seedlings in response to different HA doses.

Lateral roots and meristematic regions (Rodda et al., 2006b).

## Conclusion

Humic acids affect positively the initial growth of *B. brizantha* cv. MG5, with the best stimulus being observed when a 2.0 mM $C.L^{-1}$ dose was applied.

## Conflict of interests

The authors did not declare any conflict of interest.

**Figure 6.** Seedling growth at 0.0 (A), 1.0 (B), 2.0 (C), 4.0 (D) and 8.0 (E) mM $C.L^{-1}$ of HA.

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Vol. 14(18), pp. 1584-1589, 6 May, 2015
DOI: 10.5897/AJB2014.14203
Article Number: E38190A52714
ISSN 1684-5315
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African Journal of Biotechnology

*Full Length Research Paper*

# Effect of alkaline treatment on the sulfate content and quality of semi-refined carrageenan prepared from seaweed *Kappaphycus alvarezii* Doty (Doty) farmed in Indian waters

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Received 23 September, 2014; Accepted 22 April, 2015

**Seaweed *Kappaphycus alvarezii* previously known as *Eucheuma cottonii* is one of the best sources of kappa carrageenan and is cultivated in Philippines, Indonesia, Malaysia and other countries including India. In the present study, semi-refined carrageenan (SRC) was prepared from *K. alvarezii* with different concentrations of KOH (6, 12, 18 and 24%) at 80 ± 2°C for 2 h; its sulfate contents were 13.73±0.74, 13.66±0.27, 13.84±0.66 and 14.76±0.18%, respectively, and it was 14.10±0.34% for untreated clean weed (control). The sulfate removal with increasing concentration of KOH used for processing SRC is not statistically significant ($p < 0.05$). The KCl gel strength of untreated clean weed and 6, 12, 18 and 24% KOH treated weed was 97±10.27, 650±12.11, 637±10.25, 552±18.71 and 526±26.55 g $cm^{-2}$, respectively. Contents of 3,6-anhydrogalactose were 28.30±0.52, 33.41±0.50, 32.97±0.42, 31.15±0.60 and 31.61±0.17%. FTIR spectroscopy showed that molecules in all four SRC samples are quite similar. Spectral band was at 1257 $cm^{-1}$ which referred to ester sulfate of 930 $cm^{-1}$ for 3,6 anhydrogalactose and 848 $cm^{-1}$ assigned to galactose-4-sulfate. From the present investigation, it was observed that sulfate removal and quality improvement with increasing concentration of KOH for cooking seaweed is not statistically significant; therefore, seaweed can be subjected to lower concentration of KOH treatment (6 to 12%) to produce semi-refined carrageenan on commercial scale.**

**Key words:** Seaweed, *Kappaphycus alvarezii,* KOH treatment, semi-refined carrageenan (SRC), sulfate content, FTIR spectra, gel strength.

## INTRODUCTION

Carrageenans are sulfated linear polysaccharides extracted from certain red seaweeds of the class Florideophyceae. They have been extensively used in the food industry as thickening and gelling agent and more

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**Abbreviation: SRC,** semi-refined carrageenan; 3,6-AG, 3,6-anhydrogalacotse; FTIR, Fourier Transform Infrared Spectroscopy.

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recently used in pharmaceutical industry as excipient in pills and tablets (Campo et al., 2009; Aguilan et al., 2003). Since natural carrageenans are mixtures of different sulfated polysaccharides, their composition differs from batch to batch. Carrageenans from particular seaweed species and geographic districts differ considerably in their structure and rheological properties of solutions and gels. Therefore, the quantitative analysis of carrageenan or semi-refined carrageenan (SRC) batches is of greatest importance for industry to deliver a standard product and to develop new application based on their unique intrinsic properties. Farming of tropical seaweed *Kappaphycus alvarezii* Doty (Doty) (previously known as *Eucheuma cottonii*) in Indian water has been going on for more than a decade (Krishnan and Narayanan, 2010), and *Kappaphycus* is extensively used for the industrial production of κ-carrageenan (Glicksman, 1983; Hoffmann et al., 1993). The industrial pure carrageenan manufacturer is no longer limited to extraction of carrageenan in pure form; nowadays, SRC is used as an alternative to carrageenan. Earlier, SRC was mainly used in pet food and other applications where clarity was not an important criterion. However, recently there has been improved method to produce SRC of food grade for human consumption.

Further, the product was also processed by applying food safety procedures; therefore SRC is permitted in human consumption (Philips, 2002; Mehta et al., 2008; van de Velde et al., 2002). Two chemical transformations occur when the carrageenan containing seaweed is subjected to cooking in the presence of KOH at elevated temperature. The first transformation is desulfation, which occurs when a sulfate group bounded to the 6-position of the galactose (3,6-AG) units of a carrageenan polymer molecule is removed by the $K^+$ ions to form $K_2SO_4$ in the cooking solution. The second reaction is a dehydration of the de-sulfated product to create the recurring 3,6 anhydrous galactose polymers (Mehta et al., 2008; Christopher and Michael, 1998). It is reported that during the alkaline treatment and further washing of carrageenan bearing seaweed, alkali soluble and cold water soluble material are removed (Hoffmann et al., 1993; McHugh, 2003).

The aim of the present study was to prepare SRC with different concentrations of KOH, check the degree of desulfation with increasing concentration of KOH and observe any significant improvement in the quality of the SRC product.

## MATERIALS AND METHODS

### Sampling of *Kappaphycus alvarezii*

*K. alvarezii* fresh material was sampled from commercial farming site at Mandapam (9.28°;N 79.12°E), India; foreign matter was removed from it and then dried under sun. Dry weed samples (35% moisture) were cut into small pieces (≈1" inch), mixed well by coning and quartering method and used for preparation of SRC.

### Preparation of semi-refined carrageenan

The dry-weed sample was soaked in water for 30 min (1 part of seaweed to 6 parts of water) to remove sand and salt and given consecutive second water wash for 10 min. Then, the washed material was cooked at 80 ± 2°C for 2 h at different KOH levels (6, 12, 18 and 24%). Cooked samples were then washed with tap water at 1: 2.5 ratio (w/v) to remove the excess KOH excess and then sun-dried. The washed seaweed without KOH treatment was dried and treated as control sample. After that, the test samples were micronized into powder, and sieved through 80# mesh (180 A.S.T.M or 80 μm) to obtain SRC samples. Yield was calculated as ratio of dried SRC weight to dried seaweed weight. KOH level in spent liquor, post washes and final products of SRC were tested to see check its consumption level during SRC process. Five replications in each experiment were conducted and data were used to interpret the results.

### Quality parameter analysis

Ash content was determined gravimetrically by making ash in muffle furnace at 550°C (PNS 601:2011, Carrageenan and Processed *Eucheuma* seaweeds - Specifications). Ester sulfate content was determined using the method of sulfate hydrolysis followed by precipitation of sulfate as $BaSO_4$ (JECFA, 2010). A known amount of dried SRC (W1, g) was hydrolyzed with 50 mL of 1 N HCl for 30 min at its boiling temperature and 10 mL of 0.25 M $BaCl_2$ was dropped into it. After cooling at room temperature for 5 h, the barium sulfate precipitates were filtered using ashless filter paper and incinerated for 1 h at 700°C. The white ash was weighed as W2 and sulfate content was calculated using the equation below:

$$\% \text{ sulfate} = (W2/W1) \times 100 \times 0.4116$$

Acid insoluble matter was determined using 0.1% sulfuric acid (PNS 601:2011, Carrageenan and Processed Eucheuma seaweeds - Specifications). Sodium and potassium contents were determined by flame photometry method. The FT-IR spectra of all samples were analyzed in KBr pellets using FT-IR Spectrophotometer (Perkin-Elmer Spectrophotometer GX). Viscosity was measured at 1.5% in water at 75°C, 30 rpm and spindle no.62 using Brookfield LVDV-II+pro. KCl gel strength was determined by making 1.5% SRC in 0.2% KCl solution using Brookfield Texture Analyze, Model CT3 4500 and water gel strength was measured by preparing 1.5% SRC solution in water. The 3,6-anhydrogalactose was estimated by improved phenol-resorcinol method using fructose as standard (Yaphe and Arsenault, 1965). Microbial profile of SRC samples was carried out using the procedure as described by Jarvis et al. (1977) and Cruchaga et al. (2001).

### Statistical analysis

Statistical analyses such as analysis of variance (ANOVA, SYSTAT version 7), correlation and regression were applied to analysis the data of sulfate contents and it was considered statistically significant when $p < 0.05$.

## RESULTS AND DISCUSSION

Table 1 shows the KOH level in spent liquor, post washes and final product of SRC samples. The KOH level in spent liquor of 6, 12, 18 and 24% KOH treated material was 4.65±0.12, 9.92±0.12, 14.86±0.95 and 20.12±0.57%, respectively; similarly, the level of KOH in the first and

**Table 1.** KOH residues in spent and post wash waters and final product of semi-refined carrageenan

| Concentration of KOH used for preparation of SRC (%) | KOH residues (%) | | | |
|---|---|---|---|---|
| | Spent liquor | 1st wash water | 2nd wash water | Final Product (SRC) |
| 6 | 4.65±0.12 | 1.72±0.13 | 0.59±0.00 | 1.27±0.11 |
| 12 | 9.92±0.12 | 3.66±0.58 | 1.11±0.01 | 1.88±0.05 |
| 18 | 14.86±0.95 | 4.74±0.33 | 1.57±0.03 | 3.75±0.05 |
| 24 | 20.12±0.57 | 6.85±1.02 | 2.28±0.018 | 4.90±0.05 |

**Table 2.** Quality and Physicochemical properties of semi-refined carrageenan obtained by processing in different KOH concentration

| Parameters | Conc. of KOH used for preparation of SRC (%) | | | | |
|---|---|---|---|---|---|
| | Control | 6 | 12 | 18 | 24 |
| Yield (%); ±SD | | | | | |
| Sulfate (%); ±SD | 14.10±0.34 | 13.73±0.74 | 13.66±0.27 | 13.84±0.66 | 14.76±0.18 |
| Acid insoluble matter (%); ±SD | 13.14±0.12 | 13.87±0.08 | 13.77±0.07 | 14.50±0.08 | 14.84±0.11 |
| Ash (%); ±SD | 27.87±0.04 | 26.07±0.16 | 26.87±0.04 | 28.04±0.03 | 29.05±0.22 |
| $K^+$ (%);±SD | 6.19±0.36 | 7.12±0.10 | 10.54±0.96 | 14.91±1.02 | 17.33±1.83 |
| $Na^+$ (%):±SD | 2.15±0.04 | 0.40±0.38 | 0.32±0.04 | 0.267±0.01 | 0.25±0.01 |
| 3,6-anhydrogalactose (%); ±SD | 28.30±0.52 | 33.41±0.50 | 32.97±0.42 | 31.15±0.60 | 31.61±0.71 |
| KCl gel strength (g $cm^{-2}$) | 97±10.27 | 650±12.11 | 637±10.25 | 552±18.71 | 526±26.55 |
| Water gel strength (g $cm^{-2}$) | Nil | 310±10.33 | 286±17.19 | 235±14.4 | 224±11.82 |
| Viscosity (cP) | 104.80±0.72 | 49.95±0.21 | 34.84±0.75 | 18.67±0.54 | 13.62±0.42 |
| Total plate count (TPC) | □ 5000 | □ 2500 | □ 2500 | □ 2500 | □ 2500 |
| Yeast and mould | □ 300 | □ 100 | □ 100 | □ 100 | □ 100 |
| Salmonella and Shigella | Negative | Negative | Negative | Negative | Negative |
| Escherichia coli | Negative | Negative | Negative | Negative | Negative |

second post washes and final product increased with increasing concentration of KOH used for cooking (Table 1). Higher KOH level (>2%) in the final SRC products obtained from treating with 18 and 24% shows that further washes are required to remove the excess KOH. Table 2 depicts the quality of SRC prepared with different conc. of KOH. The yield of SRC samples obtained from treating with KOH concentration of 6, 12, 18 and 24% was 31.72±1.35, 32.55±1.35, 30.46±1.56 and 29.89±0.92% and yield of untreated material was 34.42±0.44%. Comparatively, higher yield (2.6 to 8.8%) was obtained from material treated with 12% KOH compared to low (6% KOH) and higher KOH conc. (18 and 24%). This observation is in line with the literature reported earlier (Iain, 2008). The sulfate contents of SRC prepared from 6, 12, 18 and 24% KOH were 13.73±0.74, 13.66±0.27, 13.84±0.66 and 14.76±0.18%, respectively, with 14.10±0.34% in untreated material. There are many reports on increasing level of desulfation with increasing concentration of KOH used for cooking carrageenan bearing seaweed (Sperisa et al., 2011). But in the present investigation, it was observed that sulfate removal with increasing concentration of KOH used for processing SRC is not statistically significant. The reason for more sulfate in untreated clean weed could be due to high inorganic content in the seaweed that was not removed well by pre-washing. Therefore, KOH treatment actually did not decrease the sulfate level in final product of SRC but could have been involved in intra-molecular rearrangement to form more stable structure. Percentage of each parameter in KOH treated and untreated material is shown in Table 3. No soluble fiber was found in SRC obtained from KOH treatment, but it was 3.4% in untreated material. There was no statistically significant decrease in sulfate level up to 18% KOH treatment, but it was gradual. Decrease in sodium level could be due to the replacement of potassium ion. Potassium ion increment along with sulfate level might maintain electrical neutrality of the molecule. The rise in $K^+$ content in SRC samples shows that the charge neutrality of sulfate requires $K^+$ ion. It becomes evident that the amount of positively charged cations in the studied SRC preparations slightly exceeded the quantity needed for the complete neutralization of the negative charge of the polysaccharide backbone, revealing the presence of small amount of free salts (Rando, 2009). The increase in ash content could be due to increase in inorganic matter in the molecule, therefore, no sulfate removal occurred

**Table 3.** Material balance of raw seaweed and semi-refined carragenan prepared with different KOH concentrations

| Composition | Raw seaweed (%) | Conc. of KOH used for preparation of SRC (%) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 6 | 12 | 18 | 24 |
| Moisture | 33.14±2.19 | 8.55±0.92 | 9.44 ±0.62 | 10.8±0.66 | 10.91±1.07 | 10.75±1.11 |
| Salt | 27.30±0.17 | 2.15±0.21 | 0.34±0.00 | 0.42±0.00 | 0.40±0.00 | 0.38±0.01 |
| Waste Solids | 1.92±0.12 | 0.72±0.02 | 0.14±0.00 | 0.12±0.01 | 0.11±0.00 | 0.12±0.00 |
| Soluble polymer | 5.61±0.1.20 | 3.40±0.62 | 0 | 0 | 0 | 0 |
| Fiber | 7.75±1.32 | 20.85±2.05 | 17.44±1.14 | 16.98±1.72 | 16.12±1.55 | 16.90±0.89 |
| Carrageenan | 24.28±1.88 | 64.33±3.18 | 72.64±2.67 | 71.68±2.48 | 72.46±2.81 | 71.85±2.11 |

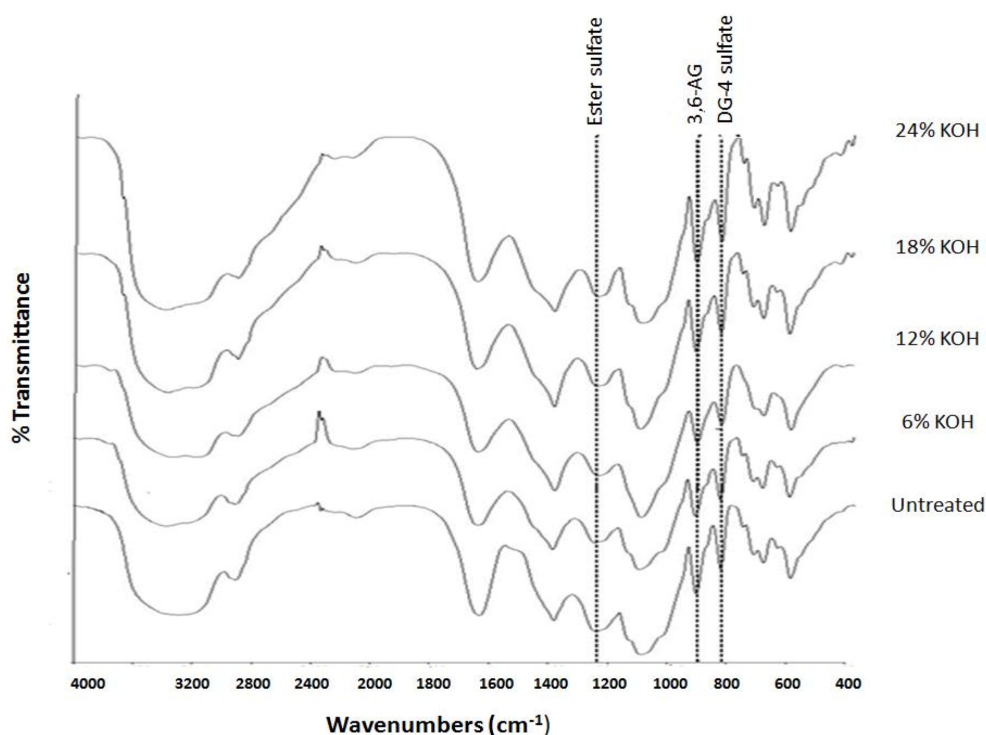


**Figure 1.** Fourier Transform Infrared Spectra of semi-refined prepared from *K. alvarezii* with different KOH concentrations.

even when cooked at higher KOH concentrations; rather, it showed higher level of sulfate which could be due to removal of alkali soluble protein and low molecular weight carbohydrates.

FTIR spectroscopy (Figure 1) shows that the molecules present in all four SRC samples are quite similar, as spectral band was at 1257 $cm^{-1}$ which referred to ester sulfate of 930 $cm^{-1}$ for 3,6-ag and 848 $cm^{-1}$ assigned to galactose-4-sulfate (van de Velde et al., 2002; Pereira et al., 2009a). Wave length ($cm^{-1}$) ranging from 1220 to 1260$cm^{-1}$ determines the presence of ester sulfate, since there is a linked bond of S-O in the ester sulfate. Wave lengths from 1010 to 1080 are assigned to glycosidic bond, while lengths from 928 to 930, 840 to 850, 825 to 830, 810 to 820 and 800 to 805 indicate the presence of 3,6-AG galactose-4-sulfate, galactose-2 sulfate, galactose-6-sulfate and 3,6 anhydrogalactose-2-sulfate, respectively (Aguilan et al., 2003; Dewi et al., 2012). It was reported that during the process of seaweeds extraction, the methods do not induce any significant change in the molecular structure of the native seaweed final product (Dewi et al., 2012). Rando et al. (2006) observed only small changes in the sulfur concentration of hybrid carrageenans extracted by water or an alkali solution, which is negligible to be considered a desulfation reaction. The 3,6-AG content KOH untreated material was 28.30±0.52% and in SRC samples obtained from treating with KOH concentration of 6, 12, 18 and 24% were 33.41±0.50, 32.97±.42, 31.15±0.60 and 31.61 ±0.71%, respectively. Therefore, it was 18.06, 16.50, 10.07 and 10.70%, respectively, more than KOH untreated material. SRC prepared with low conc. of KOH

(6%) contained higher 3,6-AG (18.06%) and it is in agreement with observation made by Rando et al. (2006). The KOH untreated material did form gel in plain water, but it formed 97±10.27g $cm^{-2}$ of KCl gel strength. KCl gel strength of SRC obtained from 6, 12, 18 and 24% KOH treatment was 570.10, 556.70, 469.87 and 442.27% respectively more than control (Table 2).

Olav et al. (1967) observed that the main effect of the alkali treatment is to transform the "intermediate" fraction into a fraction which precipitates at KCl concentrations below 0.125 M, that is, similar or identical to the χ-fraction. The authors reported that the main effect was on the "intermediate" fraction, leading to a marked increase of the 3.6-AG content and gel strength of this material. The difference between this fraction and the χ-fraction after the treatment is negligible compared to the differences between the untreated fractions. The intermediate fraction is, however, potentially a gel forming agent, in that an alkali treatment transforms it into material with approximately the same gel-forming ability of the χ-fraction from the same species (Olav et al., 1967).

Stanley (1963) investigated the carrageenan of Irish moss that monoester sulfate groups present in the carrageenan are of a highly resistant nature and have not succeeded in effecting any extensive removal of monoester sulfate groups from the carrageenan without severely de-polymerizing the polysaccharide portion of the molecule of the carrageenan. It is seemingly the case that any alkali can affect a loosening or detachment of the carbon-oxygen-sulfur bond attaching the monoester sulfate to the polysaccharide portion of the carrageenan molecule. But this reaction is completely reversible so that no extensive removal of the monoester sulfate groups can occur, except in the presence of a reagent such as barium hydroxide, which is capable of removing the liberated sulfuric acid from the reaction scene. It was used to describe the intra-molecular rearrangement that involves migration of sulfate from $6^{th}$ carbon to some other adjacent position (Stanley, 1963). Similarly, Olav et al. (1967) reported that sulfate content of carrageenan extracted from *Chondrus crispus* (Linnaeus) J. Stackhouse, *Gigartina stellata* (Stackhouse) Batters and *Gigartina skottsbergii* Setchell & N.L. Gardner before and after treatment with 1 N KOH, 100°C for 1 h cooking did not change the sulfate content much. That is, it was 32.2, 33.7, 34.0% before treatment and after treatment it was 32.0, 33.1, and 33.5%, respectively, but there was increase in 3,6-AG content of 34, 31.5 and 43.5% for *C. cripsus, G. stellata* and *G. skottsbergii*, respectively. This also proves that there is no apparent correlation between sulfate level and gel strength. Gel strength also increases 3.5 folds, 10.5 folds and 14 folds respectively for *C. cripsus, G. stellata* and *G. skottsbergii*. In the case of *Furcellaria lumbricalis* (Turner) J.V. Lamouroux, sulfate level before and after alkali treatment was 18.6 and 18.4%, respectively, showing no desulfation; but gel strength increased from 500 (untreated) to >1000 for treated weed (Olav et al., 1967). The composition of *K. alvarezii* galactan has been widely investigated. The hybrid polysaccharides from this species consist mainly of κ-carrageenan with small amounts of ι-structure. Also, minor quantities of μ-carrageenan, ν-carrageenan and G (3-linked β-D-galactopyronose), L (4-linked α-L-galacto-pyranose) and G6M,4S (3-linked 6-*O*-methyl-β-D-galacto-pyronose-4-sulfate) residues have been reported to be components of this polysaccharide (Estevez et al., 2000; 2004; Pereira et al., 2009b). The exact chemical nature, inorganic part composition and purity of the separated polysaccharides are strongly dependent on the isolation procedures. The sulfur content of the three main carrageenan types usually remains in the range of 7 to 10% for κ-carrageenan, 9.5 to 11.5% for ι-carrageenan and 11 to 13% for λ-carrageenan. Based on the idealized structure types, the theoretical (calculated) sulfur content values of the highly sulfated carrageenans appear to be somewhat higher: 8.3% for κ-carrageenan (Rando, 2009).

Extraction of carrageenans from *Eucheuma cottonii* resulted in 61% of polysaccharides during the 4 h extraction in pure water; slightly lower yields (57%) were obtained if 0.02 M KOH solution was used as the extraction medium. Somewhat, lower yields in the case of alkaline extraction media (compared to pure water) have also been reported for other *Eucheuma* species (Freile-Pelegrin et al., 2006). A slight increase in the AG content by 1.6% for furcellaran and by 2.1% for *C. truncatus* galactons during the alkaline extraction in 0.02 M KOH solution was observed (compared to the water extracted preparation), indicating the presence of alkali-labile precursor residues. In the same way AG content increases, slightly increases in case of *K. alvarezeii*. The $^{13}$C-NMR spectrum of the polysaccharide from *E. cottonii* confirmed the presence of κ-carrageenan (G4S-DA) as major component, and also indicted the presence of minor amounts of DA2S, D6S, 6S and G6M residues (I) (Rando, 2009).

Higher alkali contents in extracting media resulted in products with substantially declined molecular weight (Mw) characteristics and impaired gelling abilities. Compared to water extracted furcellaran samples gelled in salt solutions, nearly two times higher gel strength values were observed for the preparations obtained by the extraction process involving alkali metal hydroxides. This is mainly caused by the lower Mw values of the water extracted galactans, the higher content of divalent cations ($Mg^{2+}$, $Ca^{2+}$) preserving their composition and the effect of chloride ions. Compared to pure distilled water, the low yield on extraction using KOH may be due topolymer destruction. Polymer destruction produced the low molecular weight material which cannot be precipitated using alcohol (Rando, 2009). From this point of view, it can be described that sulfate removal is not a significant step and decrease in the alkali soluble matter

from the raw material increases the sulfate content indirectly. Iain (2008) reported that 15% sulfate in raw *Eucheuma* material increased to 16.6% in final product of SRC after reduction of 10% alkali soluble matter while cooking. This proves the increase in Ester sulfate content after SRC conversion, hence no significant desulfation. Therefore, the purpose of alkali treatment in the process of SRC/Carrageenan is to increase the stability of carrageenan polymer through molecular rearrangement and remove low molecular weight compounds from the seaweed.

Therefore, alkali treatment could be more of molecular reorientation process as to form a stable structure than meager sulfate removal in the process of SRC from *K. alvarezii*. From the present study, it can be concluded that the increased level of sulfate in the final SRC product could be due to removal of protein and cold water soluble low molecular weight compounds during processing. From the present investigation, it was observed that sulfate removal and quality improvement with increasing concentration of KOH for cooking seaweed is not statistically significant; therefore, seaweed can be subjected to lower concentration of KOH treatment (6 to 12%) to produce semi-refined carrageenan on commercial scale.

## Conflict of interests

The authors did not declare any conflict of interest.

## ACKNOWLEDGEMENT

The authors are very grateful to Mr. Abhiram Seth, MD, Mr. Arun Patnaik, CEO and Mr Tanmaye Seth of AquAgri Processing Private Limited, India for their constant encouragement and guidance to carry out the present investigation. The authors also wish to thank unknown reviewers of this manuscript for their critical evaluation and excellent suggestions to improve the overall quality of the manuscript.

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Vol. 14(18), pp. 1590-1595, 6 May, 2015
DOI: 10.5897/AJB2014.14178
Article Number: 6BAD6FC52716
ISSN 1684-5315
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African Journal of Biotechnology

*Full Length Research Paper*

# Modification of chitin as substrates for chitinase

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Received 11 September, 2014; Accepted 27 April, 2015

**Enzymes are able to bind to their substrates specifically at the active site. The proximity and orientation of the substrates strongly increase the likelihood that productive E–S complexes will arise. Treated chitin (powder or flake) is more efficient than crystalline chitin. This is because the latter is less active due to its insolubility. The structure of treated chitin is opened; this facilitates its interaction with the enzyme. The purpose of this research was to create a kind of modified chitin and study the characterization of the different types of chitin including functional groups by IR spectrophotometer, pore size, surface area and crystallinity by X-Ray diffraction. Chitin from shrimp shell was modified into colloidal, bead, amorphous and superfine chitin. The results of the IR spectra of colloidal and bead chitin showed a similar pattern with chitin powder; they peaked at 3447 and 3113 $cm^{-1}$ (OH and $NH_2$ groups), 1645 $cm^{-1}$ (amide groups N-H) and 1071 $cm^{-1}$ (group C-O). Superfine and amorphous chitin had similar absorbance with powder chitin but appeared to peak in the fingerprint region. Characterization of physical properties based on the pore size and surface area of powder, colloidal, superfine, amorphous and bead chitin changed the pore radius of each type of chitin due to the treatment of swelling. Crystallinity showed that specific diffractogram pattern in the three main peaks 2θ was 9.5, 19.5 and 26 with varying intensity. Chitinase activity assay using modified types of chitin substrate had higher values than chitin powder. The highest activity was in amorphous chitin with values of 1.858 U/mL. This is because it has chitin chain and the rearrangement of its structure was more open, facilitating its interaction with enzyme.**

**Key words:** Chitin modified, chitinase, substrate.

## INTRODUCTION

Chitin is a polymer that is very abundant in nature and is second only to cellulose. It is widely spread in nature as in fungi, algae, nematodes, arthropods, molluscs, plants and animals (Guo et al., 2004). So far, it has been found that it has very little large-scale industrial use because of its extreme insolubility; it cannot be absorbed or digested directly in the gastrointestinal tract (Dai, 2011).

Chitin structure has three forms, namely α, β and γ. α-Chitin is a form of a dense structure isomorphous having strong hydrogen bonds. β-Chitin has a structure with weaker intramolecular bonds but slightly more stable than α-chitin. γ-Chitin is a combination of α and β chitin structure. α-Chitin structure causes the chitin not to be soluble in the solvent, while β-chitin can be swollen in water, as

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**Abbreviations: SDS,** Sodium dodecyl sulfate; **Ct,** chitin powder; **Cc,** colloidal chitin; **Sf,** superfine; **Cb,** bead; **Ca,** amorphous.

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chitin is soluble in formic acid (Coutinõ et al., 2006). Chitin chains between each other are associated with very strong hydrogen bonds between the NH groups of one chain and the C = O groups of adjacent chains. Hydrogen bonds make chitin to be insoluble in water and to form fiber (Rostinawati, 2008). The presence of chitin in nature varies abundantly and degrades rapidly, due to the presence of some bacteria and fungi that have chitinase enzyme capable of degrading chitin. Chitin can be degraded in two lines: the first is a degradation by mechanisms that hydrolyze bonds chitinolytic 1,4-ß-glycosides, or polymers having first deacetylation and then hydrolysed by chitosanase (Herdyastuti et al., 2009).

Chitinases are extracellular inducible enzymes that catalyze the first step in chitin digestion, hydrolysis of β-1,4 linkages between the N-acetyl glucosamine (NAG) molecules. They are found in a variety of organisms including viruses, bacteria, fungi insects, higher plants and animals and play important physiological roles depending on their origin (Kuddus and Ahmad, 2013). Chitinolytic activity induces strain in the growth medium in the presence of chitin as a carbon source (Chernin et al., 1998).

Chitin can be modified by adding acid, base or detergents such as sodium dodecyl sulfate (SDS) for it to become swollen (Illankovan et al., 2007). The swelling of chitin powder process is expected to help the enzymatic reaction between chitinase and chitin. Possible modification of chitin-chitin can cause structural changes to become more open due to the restructuring of the chitin-chain. Possible rearrangement of chitin structure can cause changes in the functional group or the physical properties of each type of chitin compared with chitin powder.

## MATERIALS AND METHODS

### Preparation of chitin

Chitin was obtained from shrimp shells that have been dried and pulverized and the isolation was done by the method of Acosta et al. (1993). Chitin isolation process consists of two stages: deproteinisation and demineralization. Chitin is made into the form of colloids according to Hsu and Lockwood (1975). Chitin was dissolved in concentrated HCl (37%), and then precipitated as a colloidal suspension with the addition of cold water (5°C). The suspension was filtered and the residue was washed with distilled water until it got to neutral pH, and then dried with an oven. This process gives ± 85% recovery.

Chitin was then prepared into colloidal, superfine, bead and amorphous form. In the colloidal form, chitin is made by adding concentrated HCl (37%). Chitin beads were obtained by dissolving in 2% formic acid and 2 M NaOH solution. Chitin amorphous was prepared by dissolving chitin in a mixed solution of 40% NaOH and 0.2% SDS (which has been cooled to a temperature of 4°C). Solution was in-swell for 1 h at 4°C and matrix slurry was stored for 1 night at -20°C temperature, and then neutralized with HCl 6 N. Furthermore, it was filtered and washed with ethanol, water and acetone. The result was dried with a freeze dryer.

### Produce of chitinase enzyme

Chitinase was produced in medium with the following composition: 0.4% chitin, 0.7% $K_2HPO_4$, 0.3% $KH_2PO_4$, 0.5% $MgSO_4.5H_2O$, 0.01% $FeSO_4.7H_2O$, 0.001% $MnCl_2$ and 0.5% peptone, and incubated at room temperature for 45 h in rotary shaker at 150 rpm. The culture cells were centrifuged at 4000 rpm for 20 min (4°C). The supernatant was brought to 50% saturation with ammonium sulphate at 4°C for 30 min by stirring magnetic stirrer. The precipitate was recovered by centrifugation at 4000 rpm for 30 min (4°C) and pellet formed was solubilized in 0.1 M phosphate buffer pH 7.0. The solution was dialyzed overnight against the same buffer at 4°C.

### Chitinase assay

Chitinase activity was measured by colorimetric method based on the released N-acetyl-glucosamine(Monreal and Reese, 1969). The colloidal chitin solution (2.0 mL of 1.25% (w/v)) dissolved in 200 mM potassium phosphate buffer was added to 0.5 mL enzymes solution and incubated for 2 h at room temperature. The suspensions were centrifuged at 4000 rpm for 10 min and then supernatant (1.0 mL) was added to 2.0 mL deionized water and 1.5 mL color reagent (5.3 M sodium potassium tartrate and 3,5-dinitrosalicyclic acid 96 mM). The mixed solution was placed in boiling water for 5 min and cooled at room temperature, and then the absorbance was measured at 540 nm. One unit (U) of chitinase activity was defined as the amount of enzyme required to release 1.0 mg N-acetyl D-glucosamine from chitin per hour.

### Characterization of substrates

The structure of substrates was determined by FT-IR spectrophotometer (Perkin Elmer); the analysis of pore size and surface area was done with high speed surface area (NOVA 1200e). X-ray diffractograms were recorded by a Bruker type D 8 advance.

## RESULTS AND DISCUSSION

### Characteristic of chitin

Chitin that has been isolated from waste shrimp shells chitin powder (Ct) and has been modified into a kind of colloidal chitin (Cc), Superfine (Sf), bead (Cb) and amorphous (Ca) is as shown in Figure 1. Chitin was modified to have almost the same colour, smoother texture and lighter but more types of colloidal tawny color and form larger granules. The yield of the average obtained was 50 to 60%. The results of the analysis of the functional group on IR- spectrophotometer modified chitin (Figure 2) show the absorption at 3446 and 3113 $cm^{-1}$ (OH and $NH_2$ groups).

Sharp absorption peaked at 1645 $cm^{-1}$ indicates the presence of amide groups (N - H) and 1071 $cm^{-1}$ shows the group C - O. The spectra of Ca are a sharp peak in the fingerprint region below 700 $cm^{-1}$ which is not found in other types of chitin. Spectra of superfine showed similarity with the IR spectra of chitin and colloidal chitin. The characteristic peaks of chitin are -OH group (3433 $cm^{-1}$), -NH (amide) at 1587.54 $cm^{-1}$, CH bending vibration

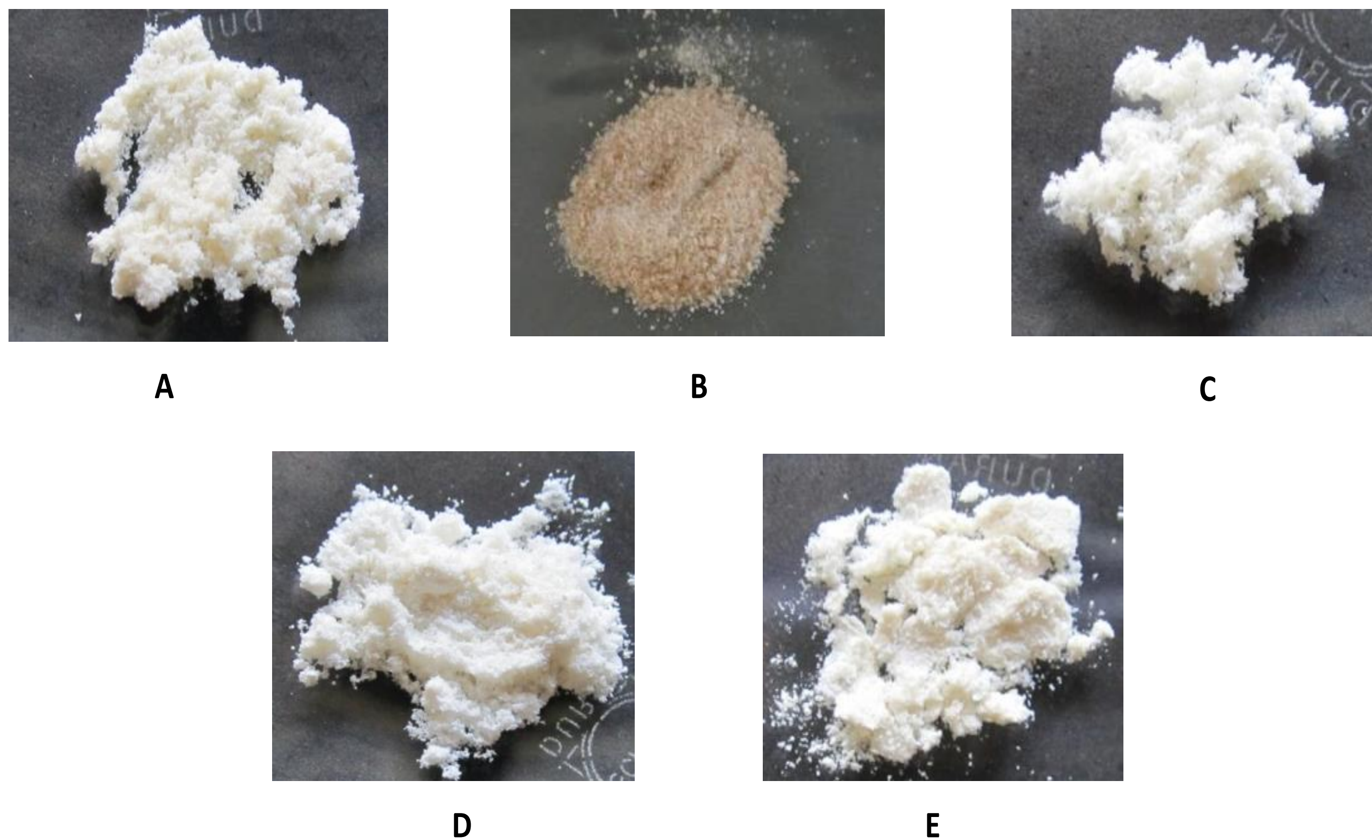


**Figure 1. (A)** Powder chitin. **(B)** Colloidal chitin. **(C)** Superfine chitin. **(D)** Amorphous chitin. **(E)** Bead chitin.

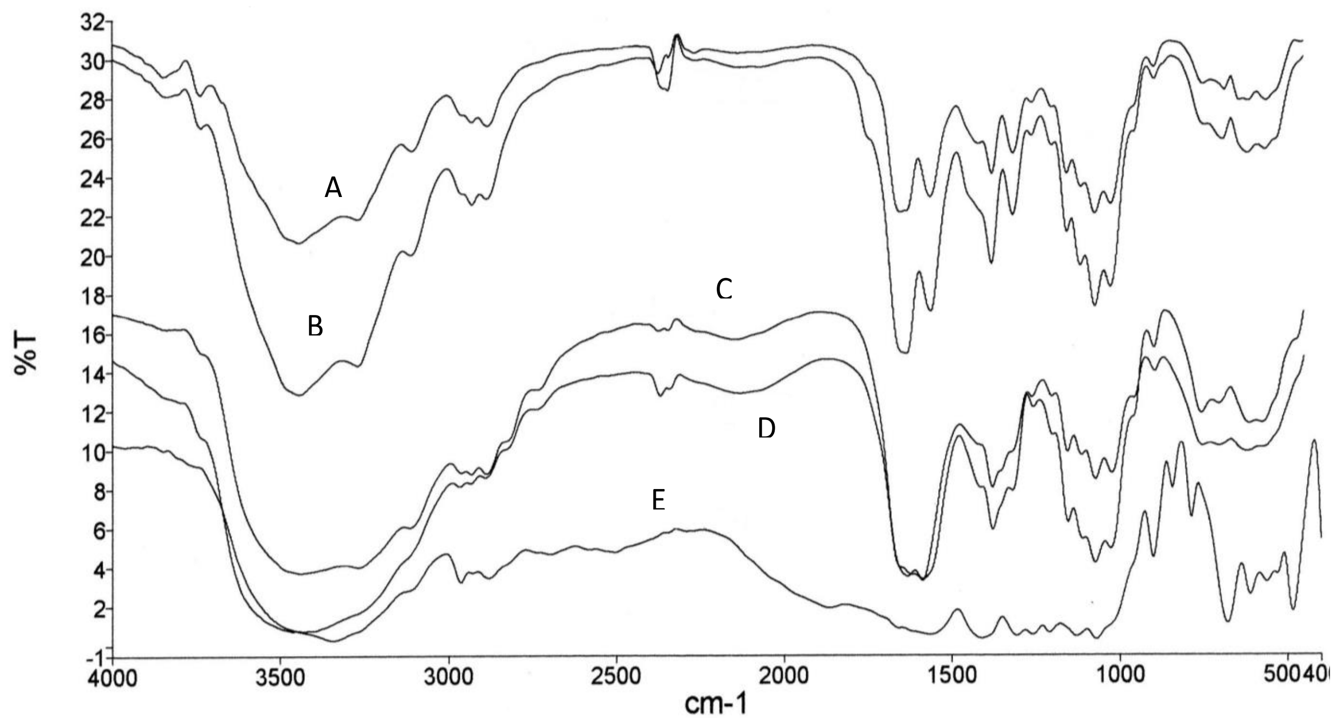


**Figure 2.** Spectra-IR of **(A)** Powder chitin, **(B)** Colloidal chitin, **(C)** Superfine chitin, **(D)** Bead chitin **and (E)** Amorphous chitin.

**Table 1.** The relevant peak FT-IR spectra of chitin substrates.

| Function group | Wave number ($cm^{-1}$) | | | | |
|---|---|---|---|---|---|
| | Ct | Cc | Sf | Cb | Ca |
| C – O | 1379 | 1380 | 1379 | 1379 | 1308 |
| N – H (bending) | 1562 | 1635 | 1567 | 1567 | - |
| C – H (streching) | 2886 | 2931 | - | - | 2963 |
| C = O | 1651 | 1635 | 1633 | 1633 | - |
| O – H | 3446 | 3446 | 3435 | 3435 | 3345 |

Ct, Powder chitin; Cc, colloidal chitin; Sf, superfine chitin; Ca, amorphous chitin; Cb, bead chitin.

**Table 2.** Analysis of physical characteristic in the chitin substrates.

| Substrate | Pore radius (A°) | Pore area ($m^2$/g) | Pore volume (cc/g) |
|---|---|---|---|
| Powder chitin | 19.108 | 1.365 | $4.00 \times 10^{-2}$ |
| Colloidal chitin | 19.044 | 2.780 | $5.00 \times 10^{-2}$ |
| Superfine chitin | 162.879 | 0.06 | 0,0001 |
| Amorphous chitin | 19.159 | 3.252 | $1.30 \times 10^{-2}$ |
| Bead chitin | 19.011 | 0.606 | $4.00 \times 10^{-3}$ |

at 1378.7 $cm^{-1}$, stretching vibration of C = O, amide -$NHCOCH_3$ (1633.1 $cm^{-1}$) and CO alcohol at 1072.9 $cm^{-1}$ (Tamimi and Herdyastuti, 2013). Table 1 shows the amides that generate elimination of carboxyl groups (Coutin˜o et al., 2006).

The results of the analysis based on the physical properties of pore size and surface area of powder, colloidal, superfine, amorphous and bead chitin are shown in Table 2. The results of the analysis showed that treatment of swelling on each type of chitin changed pore radius and became larger. Data show pore radius of superfine chitin is 9 times greater than the powder chitin. Wide pores of colloidal chitin also increased, but the chitin beads were apparently amorphous and their volume was reduced. The changes of volume size and pore radius would affect the interaction of enzymes with the substrate.

Diffractogram of chitin powder, colloidal and bead shows the same pattern. There are 3 main peaks 2θ of 9.5, 19.5 and 26; the intensity tends to be weaker in colloidal and bead chitin than in powder chitin as shown by the studies of Illankovan et al. (2007), in which the diffractogram of powder, colloidal and amorphous chitin had main peak of 2θ of 9.4 and 20.

This shows that colloidal and bead chitin has a lower degree of crystallinity than chitin powder. Swelling process in colloidal and bead chitin causes larger pores and is easily inflated in water medium; this leads to the easy interaction of enzymes with substrates than in the form of chitin powder, which is more compacted (Figure 3).

relevant peak FT-IR spectra of chitin substrates. The other research shows that chitin has -NH peak at 3269, 1663 and 1629 $cm^{-1}$ due to the reduction of primary

## Chitinase activity

Chitin from crab shell is identified as the best carbon source and colloidal chitin is reported as the best source for producing chitinase. The results show the highest activity for the amorphous chitin after colloidal chitin types (Figure 4). Suraini et al. (2008) reported that the highest specific activity produced by colloidal chitin was 14.59 U/mg. Chitinase from *Paenibacillus* sp. D1 showed that the highest activity was 35 U/mL at 30°C after 72 h (Singh, 2010). The results of optimization of culture nutrients revealed that the amount of colloidal chitin as a sole carbon source in the growth medium of *Trichoderma viride* was 32.1 U/mL (Sharaf et al., 2012). By optimizing the above cultural conditions, the production of chitinase from *Bacillus amyloliquefaciens* SM3 increased by three fold to 33.5 U/mL at the final stage (Das et al., 2012). This form of chitin is tight due to its anti-parallel chain form and it stabilizes polymorphism shape naturally causing chitin not to dissolve in the solvent (Majtán et al., 2007). Modified chitin using SDS detergent leads to swelling of chitin structure causing changes in the physical properties of chitin powder. Amorphous type chitin has fingers longer than other types of chitin and three times larger area than the chitin powder. The data indicate that amorphous chitin is more open and more

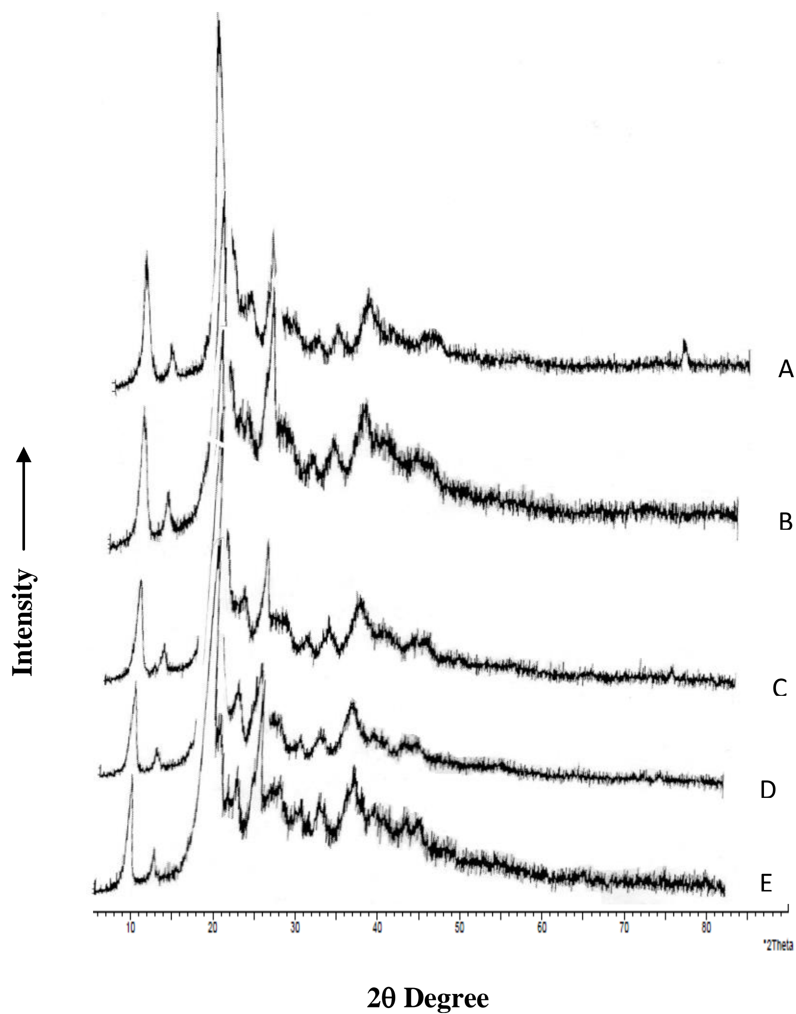


**Figure 3.** Difractograme of powder chitin (A), colloidal chitin (B), amorphous chitin (C), bead chitin (D) and superfine chitin (E).

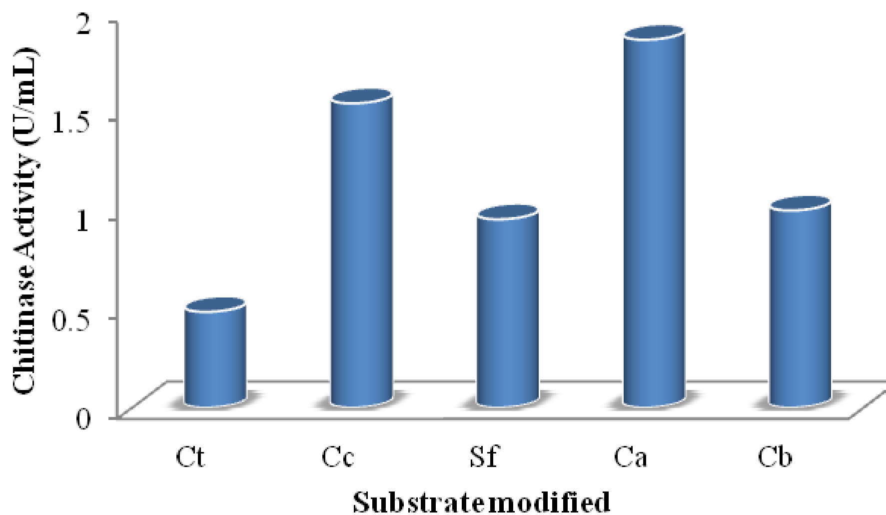


**Figure 4.** Chitinase activity with substrate types. Ct, Powder chitin; Cc, colloidal chitin; Sf, Superfine chitin; Ca, amorphous chitin; Cb, bead chitin.

likely to facilitate interaction with chitinase that can provide higher chitinase activity than other substrates. Ilankovan (2005) reported that among the chitinolytic activities of the commercial enzymes investigated with amorphous chitin as substrate, the bovine pepsin had the highest chitinolytic activity.

## Conclusion

Modification of chitin by adding detergents causes characteristic changes in its physical properties and the structure becomes more open than chitin powder, thus causing its interaction with the enzyme chitinase. Amorphous chitin can be used as an alternative substrate or inducer for chitinase enzyme indicated by higher chitinase activity than using chitin powder.

## Conflict of interests

The authors did not declare any conflict of interest.

## ACKNOWLEDGEMENTS

The research was supported by Ditlitabmas, DP2M-DIKTI, National Education Department of Indonesia. We are also grateful to Mizan Tamimi and Adi Wirawan who indeed rendered assistance in this research.

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Vol. 14(18), pp. 1596-1604, 6 May, 2015
DOI: 10.5897/AJB2014.14327
Article Number: A822FE552717
ISSN 1684-5315
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http://www.academicjournals.org/AJB

African Journal of Biotechnology

*Full Length Research Paper*

# Isolation of microalgae species from arid environments and evaluation of their potentials for biodiesel production

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Received 19 November, 2014; Accepted 22 April, 2015

**Twenty-five (25) strains of microalgae were isolated and screened for growth, lipid accumulation and biodiesel production from arid environments of North East Nigeria. Isolates that produced biomass concentration (≥ 1.50 g $L^{-1}$ cell dry weight), accumulated high concentrations of lipids (≥ 18% of the cell biomass) and could be purified on agar plates were selected for further studies. Four strains morphologically identified as *Oocystis* IA1, *Chlorella* IA7, *Chlorococcum* KA9 and *Botyrococcus* YA5 were selected. The maximum biomass concentrations (g $L^{-1}$) and lipid contents (%) were 3.02, 3.92, 3.59 and 2.89, and 57, 67.23, 63 and 65 dry cell weights for *Oocystis* IA1, *Chlorella* IA7, *Chlorococcum* KA9 and *Botyrococcus* YA5, respectively. The highest oil productivities (mg $L^{-1}$ $day^{-1}$) were 135.28 ± 3.32 (*Oocystis* IA1), 165.22 ± 3.36 (*Chlorella* IA7), 131.76 ± 1.11 (*Chlorococcum* KA9) and 140.37 ± 2.13 (*Botyrococcus* YA5). Maximum chlorophyll contents (g $g^{-1}$ cell) were 34.97, 30.00, 39.71 and 32.27, respectively. Fatty acid methyl ester profiles indicated the presence of C16:0, C16:1, C18:0, C18:1, and C18:2. Oleic acid (C18: 1) was predominant, ranging between 73.3 and 85.6%. Biodiesel properties were within the ASTM standards. The present study suggested that the four isolates are good for biodiesel production.**

**Key words:** Biodiesel, *Chlorella*, *Chlorococcum*, *Botyrococcus* sp., lipid accumulation, microalgae, *Oocystis*.

## INTRODUCTION

There is a perceptible problem of oil crisis in Nigeria and many other parts of the world. Researchers have postulated that fossil fuel consumption would rise by about 60% in the next 25 years (Rittmann, 2008). Veziroglu and Sahin (2008) also noted that fossil fuel will deplete significantly over the next few decades. In addition, fossil fuel is associated with global warming and climate change. They are non-renewable, cause pollution, and their prices steadily increase in many countries of the world. Renewable energy, carbon neutral

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**Abbreviation: FAME,** Fatty acids methyl esters.

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and environmentally friendly alternative sought globally is biofuel energy. In this respect, first-generation biofuels made from sugar, starch, vegetable oil or animal fats using conventional technology were practical alternatives. This is however being criticized for diverting food away from human food chain, leading to food shortages and price rises (Rosegrant et al., 2006). In addition, to generate enough biofuel from arable crops that could serve any country for one year, available land area would not support such production (Chisti, 2007). The second-generation biofuel made from the stalks of wheat, corn, and wood, generally referred to as non-food-crops from cellulosic materials are also being explored in search of replacement of fossil fuel with their attendant demerits. The major disadvantage as noticed by Schenk et al. (2008) is the economic non-feasibility and environmental related problems.

Microalgal lipids are potential sustainable biofuel feedstocks in future (Chisti, 2007). The use of microalgae as biofuel feedstock has many advantages. They have rapid growth rate with cell doubling time of 1 to 10 days (Schenk et al., 2008) in some species. They have high lipid content - more than 50% of cell dry weight (Hu et al., 2008). Karpenyuk et al. (2013) reported 80% oil in some microalgae and Metting (1996) reported even 90%. Microalgae need smaller land usage. They have 15 to 300 times more oil production than conventional crops on a per-area basis (Li et al., 2010). They can grow on saline and hyper-saline water, and thus there is less dependency on freshwater (Sing et al., 2013). They have high carbon sequestration rate (Jorquera et al., 2010); Moheimani et al., 2012). Algal biomass production systems can easily be adapted to various levels of biotechnological skills (Vonshak, 1990).

Microalgae-based biofuel however has its own bottlenecks. Biodiesel oil productivity by microalgae is determined by the overall growth rates of the algae and the design of the reactors. In addition, the ability of the algae to efficiently harvest light energy for the synthesis of oil is another major consideration in oil productivity: Further Particulars Algae.doc - SlideShare. (n.d.). Retrieved from http://www.slideshare.net/Pammy98/furtherparticularsalgaedoc. Although, many strains of microalgae have been isolated from various environments and reported to have good potentials for biodiesel oil production, the present cost of microalgae diesel oil is still too high to compete with fossil diesel. There is therefore a need to screen for more oil productive strains.

Moreover, it is desirable to locate microalgae culture ponds in arid areas that are not suitable for conventional agriculture to reduce competition for arable land. In that regard, it is necessary to isolate oil productive strains from such environment since such strains could be better adapted to the arid climates. In this work, therefore, effort was made to isolate oil-producing strains from arid region of North East Nigeria.

## MATERIALS AND METHODS

### Microalgae and culture condition

Microalgae were obtained from different locations around North East Nigeria from water bodies between June and December 2009 and were cultivated in BG-11 medium. The medium contains $NaNO_3$ 1.5 g, $K_2HPO_4.3H_2O$ 0.04 g, $KH_2PO_4.3H_2O$ 0.2 g, disodium EDTA 0.001 g, Fe ammonium citrate 0.001 g, citric acid 0.006 g, $Na_2CO_3$ 0.02 g and 1 ml of trace metal solution per litre, pH 7.3. The trace metal solution contains $H_3BO_3$ 2.85 g, $MnCl_2$. $4H_2O$ 1.8 g, $ZnSO_4.7H_2O$ 0.02 g, $CuSO_4.5H_2O$ 0.08 g, $CoCl_2$ $.6H_2O$ 0.08 g and $Na_2MoO_4.2H_2O$ 0.05 g per litre. Each isolate was cultivated in a 75 ml sterile medium contained in a 200 ml capacity sterile transparent Roux bottle capped with urethane foam. Pond water containing microalgae was inoculated (inoculum ratio = 25%) and incubated near windows in our Laboratory at room temperature (30±2°C) under atmospheric $CO_2$. The incubation lasted between two and three weeks depending on the isolate. Purification of the isolates involved successive decantation of the upper growing layer into a freshly prepared medium followed by plating on the BG-11 medium solidified with 1% agar-agar. Growth on the agar plates lasted also for about 3 weeks for some of the cultures. The emergent colonies were re-inoculated into a sterile BG-11 agar medium with repeated sub-culturing. Thereafter, the colonies were transferred into a fresh sterile BG-11 medium.

### Microscopic identification of the isolates

Microscopic identifications of the isolates were based on cell morphology and colonial characteristics (APHA, 1985). Cell micrographs were prepared using a Microscope Digital Camera model DCM310.

### Experimental set-up for the growth of the isolates

Experimental set-up for the growth of the isolates involved cultivation of each isolate in a 2000 mL transparent Teflon bottle containing 1000 mL of sterile BG-11 medium at an initial pH of 7.3. Each bottle was inoculated with one week-old pure culture (inoculum ratio = 15%), capped with urethane foam and incubated at room temperature (30±2°C) for 12 days. They were either naturally illuminated (about 4000 lux) or by using 2-feet fluorescent tubes arranged in parallel with maximum light intensity of 5000 lux. The photoperiod was 14 h (14 h light followed by 10 h dark). The light intensity was measured at the centre of the culture bottle with a digital light meter (model LX-1000, Custom Limited, Japan). All the cultures were shaken twice at 120 rpm for 3 min in a Gallenkamp orbital shaker (Gallenkamp Limited, UK) every day and samples were taken every two days for analyses. All the experiments were carried out in triplicates and the results presented as average values ± standard deviation.

### Analytical procedures

#### *Cell dry weight*

Samples were harvested by centrifugation at 3,000 rpm in a bench-top centrifuge for 15 min and washed three times with distilled water. This was thereafter transferred to a pre-weighed filter paper ($w_1$) and dried to a constant weight in a hot air oven at 70°C overnight. They were left in desiccators for 5 h before weighing ($w_2$).

$$\text{Cell concentration (g/L)} = \frac{W2 - W1}{V} \times 1000$$

Where, $w_2$ = weight of filter paper and dried cells (g), $w_1$ = weight of filter paper (g), v = volume of culture (ml).

### *Measurement of chlorophyll content*

The chlorophyll contents were measured according to the procedure reported by Becker (1994) using methanol and water.

### *Lipids extraction*

Lipids were extracted in a chloroform-methanol-water system by the two-step method of Bligh and Dyer (1959). The lipid fraction was transferred into a pre-weighed vial. The chloroform was evaporated by heating in a 55°C water bath under a constant stream of nitrogen gas. After 1 h in a 105°C oven, vials were weighed again. The extracted lipid was expressed as % lipid. Lipid productivity was calculated as the product of average lipid content and biomass productivity in gram per litre per day (Griffiths and Harrison, 2009).

$$\text{Plipids}\ (\text{gL}^{-1}\text{d}^{-1}) = \frac{\text{Total microalgae biomass production (g)} \times \text{lipid content (\%)}}{\text{working volume (l)} \times \text{cultivation time}}$$

### *Transesterification of the microalgal oil*

Transesterification of the microalgal oil was done according to the methods reported by Kywe and Oo (2009) and Ojolo et al. (2011), using methanol (6:1) and 1% NaOH as the catalyst. The reaction temperature was 65°C while the reaction time was 1 h.

### *Fatty acids methyl esters (FAME) analyses*

The fatty acids profiles were determined using Shimadzu Gas Chromatograph (Shimadzu, Japan, Model GCMS-QP2010 Plus). The analytical method involved the following details. Number of rinses with both pre and post solvents were 4 each; number of rinses with sample was 3; plunger speed (suction, injection) and syringe insertion were high. The injection mode was normal, pumping times was 4, and injection port dwell time was 0.3 s. There was no terminal air gap, plunger-washing speed was high, washing volume was 0.8 µL, and both syringe suction position and syringe injection position were 0.0 mm. The column oven temperature was 70°C, injection temperature was 250°C, injection mode was split, and the flow control mode was linear velocity while the pressure was 116.9 kPa. Total flow was 40.8 mL/min, column flow was 1.80 mL/min, linear velocity was 49.2 cm/s and purge flow was 3.0 mL/min while split ratio was 20.0. High-pressure injection, carrier gas saver and splitter hold were all turned off. The GC program of the GCMS-QP2010 Plus ion source temperature was 200.0°C, interface temperature was 250°C, solvent cut time was 2.50 min, detector gain mode was relative and detector gain was 0.00 kV while threshold was 2000. The MS table start time was 3.00 min, end time 24.00 min, ACQ mode was scan, event time was 0.50 s, scan speed was 666 and start m/z was 30.00 while end m/z was 350.00.

### *Properties of the biodiesel from the microalgal oils*

To determine the properties of the microalgal methyl esters, the volume of the algal methyl ester was measured in a graduated cylinder and density obtained by dividing the mass by the volume. Viscosity was measured with a viscometer (Anton Paar DSA 3000 M Stabinger Viscometer instrument). The kinematic viscosity was calculated from the equation , where v was the kinematic viscosity, µ the dynamic viscosity, and ρ the density. A crucible, a thermometer and a hot plate were used for flash point determination. The acid value of the biodiesels was measured by AOCS Method (Ca 5a-40). Alcoholic KOH (0.5 M) was standardized using 1 M $H_2SO_4$. An aliquot (1 g) of biodiesel was weighed into a conical flask, followed by the addition of 10 ml of hot neutralized ethanol and the mixture boiled on a water bath. The solution was then titrated whilst hot to neutrality with 0.5 M alcoholic KOH using phenolphthalein indicator. The titre values were taken after triplicate runs, the mean value recorded, and their acid values calculated using a conventional formula.

$$\%\text{FFA (as oleic)} = \frac{\text{ml alkali} \times \text{N of alkali} \times 28.2\ \text{mg}}{\text{sample weight (g)}}$$

$$\text{acid value} = \%\text{FFA (as oleic)} \times 1.99$$

Acid value conversion factors for lauric and palmitic were 2.81 and 2.19, respectively. The iodine values determined by AOAC (1990) Method 920.159 (18) required tetrachloromethane, potassium iodide, sodium thiosulphate, and Wij's solution. An aliquot (0.15 g) of the biodiesel was weighed into a conical flask followed by the addition of 15 ml of tetrachloromethane and 25 ml of Wij's solution. This mixture was then placed in a stoppered conical flask, swirled gently and placed in a dark cupboard for 1 h after which 20 ml of potassium iodide solution and 150 ml of distilled water was added. After gentle shaking, liberated iodine was titrated with 0.1 M sodium thiosulphate solution until a yellow colour appeared. The starch indicator (1 ml) was added and titration continued until the blue colour disappeared even after vigorous shaking. The titre values were taken after triplicate runs and the mean values recorded. Thereafter, a blank was prepared in which distilled water was added in place of biodiesel. The iodine value was calculated according to the formula below:

$$\text{iodine value} = \frac{(\text{B} - \text{S}) \times \text{N} \times 12.69}{\text{sample weight (g)}}$$

Where, S = sample titration, B = blank titration, N = normality of $Na_2S_2O_3$ solution, 12.69 was used to convert from meq. thiosulphate to g iodine; M. W. iodine is 126.9.

Determination of the peroxide values of the biodiesels was done according to AOAC (1990) method 965.33 (18). An aliquot (1 g) of biodiesel was weighed into a conical flask containing 6 ml of glacial acetic acid: chloroform solution (3:2 v/v). Saturated potassium iodide solution (0.1 ml) was added and the solution swirled in the dark for 1 min after which 6 ml of distilled water was added. The mixture was titrated with 0.01 M sodium thiosulphate with vigorous shaking until all the yellow colour disappeared. Thereafter, starch indicator (0.1 ml) was added and titration continued until all the blue colour disappeared. The above procedure was repeated for the various samples. A blank experiment was carried out in which 1 ml of distilled water was added in place of biodiesel. The titre values for the various samples and the blank were determined after each triplicate runs and the mean values recorded. These were used to calculate the peroxide values of the biodiesels according to the formula:

$$\text{Peroxide value} = \frac{(\text{S} - \text{B}) \times \text{N} \times 1000}{\text{sample weight (g)}}$$

Where, S = sample titration, B = blank titration and N = normality of $Na_2S_2O_3$ solution.

Saponification values of the oils were determined according to AOAC (1990) Method 920.160 (18). A 1.0 g quantity of the algal diesel was weighed into a conical flask containing 12.5 ml of 0.5 M alcoholic KOH and the mixture refluxed for 30 min. The reflux condenser was removed and the reflux mixture titrated with 0.5 M $H_2SO_4$ using phenolphthalein indicator. A blank experiment was also titrated without adding the oil and without refluxing. The titre values for the sample extracts and the blank were taken each after triplicate runs and the mean values recorded. These were used to calculate the saponification values of the oils according to the formula:

$$\text{saponification number} = \frac{(\text{S} - \text{B}) \times \text{N} \times 56.1}{\text{sample weight (g)}}$$

Where: S = sample titration, B = blank titration, N = normality of the HCl, 56.1 = the M. W. of KOH.

#### Statistical analysis

Statistical analysis was by Multiple-Sample Comparison using STATGRAPHICS Centurion XVI Version 16.1.05 (32-bit). All the experiments were carried out in triplicates and data were presented using descriptive statistics.

## RESULTS AND DISCUSSION

### Screening of the isolates for growth and lipid accumulation

All the 25 isolates grew on BG-11 medium (Table 1) with variation in growth and lipid accumulation rates. Isolates AA7, GA8, GA10, IA1, IA7, IA8, KA9, UA11, UA8 and YA5 grew well and produced biomass concentrations greater than or equal to 1.50 g $L^{-1}$ dry cell weight. Isolates GA1, GA8, GA12, IA1, IA2, IA6, IA7, IATM, KA9 and YA5 grew and had lipid contents up to 18% of the cell biomass (Table 1). The predominant fatty acid composition of each isolate is oleic acid (C18.1). Out of the 25 isolates, sixteen (AA3, AA7, GA1, IA1, IA2, IA6, IA7, IA8, IA9, IA25, IAMAN, KA9, KA11, UA8, UA11 and YA5) could be purified on agar plates (Table 1). While isolates GA8, GA10 IA8 UA11, UA8 and AA7 showed good growths, they had insignificant lipids. Isolate GA1 had significant lipid but showed relatively low growth. The most effective lipid producers that showed good growth and could be purified on agar plates, were morphologically identified as *Oocystis* IA1, *Chlorella* IA7, *Chlorococcum* KA9 and *Botyrococcus* YA5 and were used for further studies (Figure 1). The microalgae *Oocystis* IA1, *Chlorella* IA7, *Chlorococcum* KA9 and *Botyrococcus* YA5 were superior to other isolates of this same study on the ground of sustained growth and lipid accumulating capabilities. During the screening processes, some of the isolates did not show good growth in the media. Some did not withstand the isolation conditions and showed orange to brownish coloration during their cultivation and subsequently bleached and died. Some isolates had insignificant lipid accumulating capabilities whereas purification of some on agar plates was difficult. Isolation and screening of native species for biodiesel production are important because they are already adapted to their local environment (Wu et al., 2012).

### Growth of the four isolates on BG-11 medium

The cultures attained the stationary phase between 10th to 12th days of incubation (Figure 2). The overall highest biomass concentration of 3.92 g $L^{-1}$ was obtained by *Chlorella* IA7 on the 10th day of cultivation (Figure 2). *Chlorococcum* KA9 also had its highest biomass of 3.55 g $L^{-1}$ on the 10th day of cultivation. The highest biomass concentrations for *Oocystis* IA1 and *Botyrococcus* YA5 were 3.02 and 2.89 g $L^{-1}$, respectively (Figure 2).

### Comparison of biomass concentrations, oil contents, productivities and chlorophyll contents of the isolates

Comparisons of maximum biomass concentrations, oil contents, productivities and chlorophyll contents of the isolates are presented in Table 2. The highest oil productivity of 165.22 ± 3.36 mg $L^{-1}$ $day^{-1}$ was achieved by *Chlorella* IA7. The highest oil productivities obtained for *Oocystis* IA1, *Chlorococcum* KA9 and *Botyrococcus* YA5 were 135.28 ± 3.32, 131.76 ± 1.11 and 140.37 ± 2.13 mg $L^{-1}$ $day^{-1}$, respectively (Table 2). Maximum lipid content got from *Oocystis* IA1, *Chlorella* IA7, *Chlorococcum* KA9 and *Botyrococcus* YA5 were 57, 67.23, 63 and 65% dry cell weights, respectively (Table 2). Maximum chlorophyll contents (g $g^{-1}$ cell) of the isolates were 34.97, 30.00, 39.71 and 32.27 for *Oocystis* IA1, *Chlorella* IA7, *Chlorococcum* KA9 and *Botyrococcus* YA5, respectively. For the production of biodiesel from microalgae, biomass and lipid contents and compositions remain critical issues (Chisti, 2007; Griffiths and Harrison, 2009). The present study suggests that *Chlorella* IA7 was the best candidate for high-density culture and lipid productivity followed by *Botyrococcus* YA5 while *Chlorococcum* KA9 had the highest chlorophyll content (Table 2). Mata et al. (2010) reported lipid content value of 10.5% for *Oocystis pusilla*, which is lower than that obtained in the present work for *Oocystis* sp. *Chlorella* IA7 of the present study, with its final biomass of 3.92 g $L^{-1}$ was higher than 2.58 ± 0.07 g $L^{-1}$ reported by Praveenkumar et al. (2012), and 1.65 ± 0.07 reported for *Chlorella vulgaris* (Abou-Shanab et al., 2011). The final biomass concentration reported by Cheirsilp and Torpee (2012) for marine *Chlorella* sp was supported by the present study, however, the final lipid content and productivity varied.

Similarly, the final lipid productivity of *C. vulgaris* ESP-31 (144 mg $L^{-1}$ $d^{-1}$) was lower but biomass concentration of 5 g $L^{-1}$ (Yeh and Chang, 2012) was higher than that

**Table 1.** Screen characteristics of the isolates for growth and lipid accumulations.

| Isolate designation | Max. cell dry weight g $L^{-1}$ | Isolation source | Lipid content (%) | Predominant fatty acid composition | Purification on agar plate | Cultural characteristics |
|---|---|---|---|---|---|---|
| AA3 | 1.65 | Fresh water | 8.65 | C18:1 | + | Long cylindrical and striated |
| AA7 | 1.88 | Fresh water | 10.26 | C18:1 | + | Long cylindrical and striated |
| ALS | 0.22 | Fresh water | 10.21 | C18:1 | - | Long cylindrical and striated |
| AUSA1 | 1.00 | Fresh water | 7.14 | C18:1 | + | Motile, Euglenoid |
| GA1 | 1.40 | Fresh water | 18.36 | C18:1 | + | Coccoidal |
| GA8 | 1.60 | Fresh water | 19.92 | C18:1 | - | Coccoidal |
| GA10 | 2.20 | Fresh water | 15.32 | C18:1 | - | Coccoidal |
| GA12 | 0.84 | Fresh water | 20.29 | C18:1 | - | Coccoidal |
| IA1 | 2.46 | Fresh water | 26.18 | C18:1 | + | Oval, conspicuous nucleus |
| IA2 | 0.30 | Fresh water | 20.00 | C18:1 | - | Coccoidal |
| IA6 | 1.00 | Fresh water | 26.13 | C18:1 | + | Coccoidal |
| IA7 | 1.87 | Fresh water | 45.59 | C18:1 | + | Oval, conspicuous nucleus |
| IA8 | 2.55 | Fresh water | 11.20 | C18:1 | + | Coccoidal |
| IA9 | 1.36 | Fresh water | 8.07 | C18:1 | + | Coccoidal |
| IA11 | 1.00 | Fresh water | 10.12 | C18:1 | - | Coccoidal |
| IA25 | 1.60 | Fresh water | 12.14 | C18:1 | + | Coccoidal |
| IAMAN | 1.40 | Fresh water | 14.35 | C18:1 | - | Coccoidal |
| IATMU | 0.80 | Fresh water | 19.62 | C18:1 | + | Coccoidal |
| KA9 | 1.62 | Fresh water | 31.60 | C18:1 | + | Coccoidal, motile and minute |
| KA11 | 1.00 | Fresh water | 18.61 | C18:1 | + | Long cylindrical and striated |
| UA8 | 1.19 | Fresh water | 7.95 | C18:1 | + | Long cylindrical and striated |
| UA11 | 1.93 | Fresh water | 9.30 | C18:1 | + | Coccoidal |
| UOA4 | 1.18 | Fresh water | 10.33 | C18:1 | - | Coccoidal and motile |
| UOA8 | 1.00 | Fresh water | 17.82 | C18:1 | - | Motile, Euglenoid |
| YA2 | 1.00 | Fresh water | 16.96 | C18:1 | - | Circular |
| YA5 | 1.80 | Lake chad | 47.82 | C18:1 | + | Coccoidal and motile |

- = could not be purified; + = could be purified on agar plate.

obtained in the present study. Liu et al. (2011) reported a range of dry biomass (g $L^{-1}$) of 0.55±0.21 to 5.75±0.45, lipid content (%) of 18.67±2.94 to 52.08±2.37 and lipid productivity (mg $L^{-1}$ $d^{-1}$) of 13.74±2.94 to 194.27±1.56 for *Chlorella* sp which were corroborated by the present study.

In addition, Mata et al. (2010) reported a maximum lipid content and productivity for different strains of *Chlorella* sp. as 63.0%, and 121.4 mg $L^{-1}$ $d^{-1}$, which were similar to the values in the present study. Moheimani (2013) reported maximum biomass and lipid productivity of 407±5.5 mg biomass $L^{-1}$ $day^{-1}$ which was higher than the values of the present study and 99±17.2 mg lipid $L^{-1}$ $day^{-1}$ for a *Chlorella* sp.

Liu et al. (2011) and Mata et al. (2010) reported lower values of biomass productivity, lipid content and lipid productivity for *Chlorococcum* sp than the value of the present study. Report by Mata et al. (2010) for the range of lipid content obtained for *Botryococcus braunii* (25.0 to 75.0) was similar to the values obtained in the present study.

However, the final cell dry weight of 4.55 g $L^{-1}$ obtained by Zhang et al. (2011) for *B. braunii* was higher than the 3.2 g $L^{-1}$ of the present study. Similarly, Ruangsomboon (2012) obtained lipid content for *B. braunii* which was supported by the present work, however; the highest biomass concentration of 1.91 g $L^{-1}$ (Ruangsomboon, 2012) was lower than that obtained in the present study.

**(a)** ***Oocystis*** **IA1**

**(b)** ***Chlorella*** **IA7**

**(c)** ***Chlorococcum*** **KA9**

**(d)** ***Botyrococcus*** **YA5**

**Figure 1.** Photos of the representative isolated microalgae (a – d).

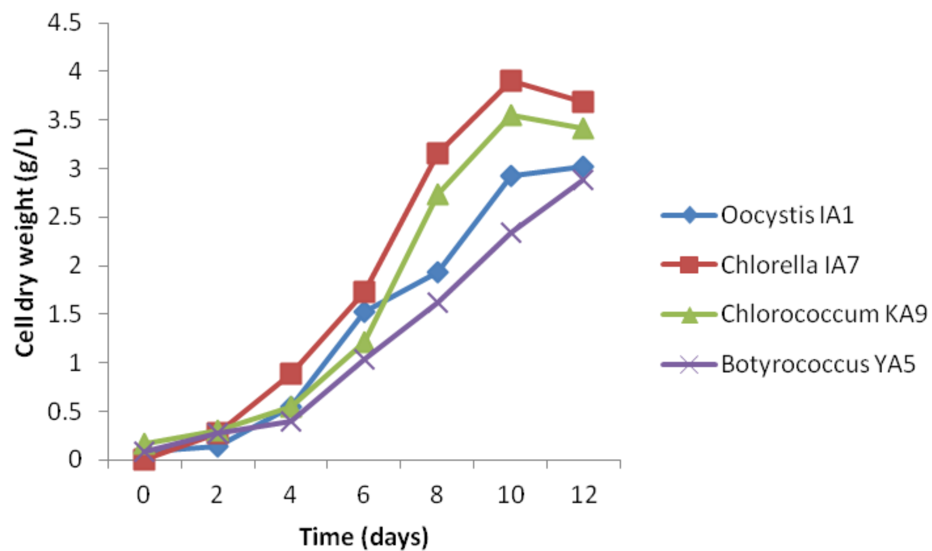


**Figure 2.** Growth curve of the four isolates on BG-11 medium.

**Table 2.** Comparison of maximum biomass concentrations, maximum oil contents and productivity and maximum chlorophyll contents of the isolates.

| Isolate | Max. Chlorophyll a + b (mg/g cell) | Specific growth rate $\mu(d^{-1})$ | Maximum biomass concentration ($gL^{-1}$) | Biomass productivity ($gL^{-1}day^{-1}$) | Max. oil content (%) | Max. oil productivity ($mgL^{-1}day^{-1}$) |
|---|---|---|---|---|---|---|
| *Oocystis* IA1 | 34.97 | 0.37 ± 0.02 | 3.02 ± 0.20 | 0.48 ± 0.07 | 57.00 ± 1.15 | 135.28 ± 3.32 |
| *Chlorella* IA7 | 30.00 | 0.44 ± 0.02 | 3.92 ± 0.10 | 0.65 ± 0.03 | 67.23 ± 3.99 | 165.22 ± 3.36 |
| *Chlorococcum* KA9 | 39.71 | 0.35 ± 0.01 | 3.59 ± 0.19 | 0.60 ± 0.01 | 63.00 ± 3.32 | 131.76 ± 1.11 |
| *Botyrococcus* YA5 | 32.27 | 0.32 ± 0.01 | 2.89 ± 0.03 | 0.44 ± 0.10 | 65.00 ± 2.72 | 140.37 ± 2.13 |

**Table 3.** GCMS analyses results showing fatty acid methyl ester (FAME) profiles of the four microalgae.

| Sample designation | Lauric acid C12:0 | Myristic acid C14:0 | Palmitic acid C16:0 | Stearic acid C18:0 | Oleic acid C18:1 | Linolenic acid C18:3 | Elaidic acid C19: | Erucic acid C22:1 |
|---|---|---|---|---|---|---|---|---|
| *Oocystis* IA1 | 0.80 | 5.12 | - | 9.34 | 76.90 | 1.21 | - | 2.12 |
| *Chlorella* IA7 | - | 4.40 | - | 8.26 | 84.31 | 1.15 | - | 0.96 |
| *Chlorococcum* KA9 | 5.4 | 0.6 | - | 9.69 | 73.15 | 1.58 | - | 3.64 |
| *Botyrococcus* YA5 | - | 3.66 | 7.48 | - | 85.64 | - | 1.25 | 1.91 |

## Fatty acid methyl ester (FAME) profiles of the four microalgae

The main fatty acids in *Oocystis* IA1 were myristic acid (C14:0), stearic acid (C18:0), oleic acid (C18:1) and linolenic acid (C18:3). Among these, stearic acid and oleic acid were the two most predominant fatty acid components, accounting for over 80% of the total fatty acids present in the microalgal lipids (Table 3). For *Chlorella* IA7, the main fatty acids were myristic (C14:0), stearic acid (C18:0), oleic acid (C18:1) and linolenic acid (C18:3). Among these, stearic acid and oleic acid were the two most predominant, accounting for up to 90% of the total fatty acids in the alga. *Chlorococcum* KA9 contained, in addition, lauric and erucic acid. However, the predominant fatty acids were stearic acid and oleic acid accounting for about 82% of the total fatty acids in the alga methyl ester. *Botyrococcus* YA5 contained predominantly palmitic and oleic acids (Table 3). Six different fatty acids were found in *Oocystis* IA1 and *Chlorococcum* KA9 methyl esters; whereas, *Chlorella* IA7 and *Botyrococcus* YA5 methyl esters contained five fatty acids each. Similar fatty acid compositions were observed in the four microalgal methyl esters of this study. The predominant fatty acid was oleic acid (C18: 1) signifying that the isolates are appropriate for the production of excellent quality biodiesel. Oleic acids are the markers of biodiesel quality (Yoo et al., 2010). The composition and structure of fatty acid esters determine the properties of biodiesel and addition of methyl oleate improves its properties such as its oxidative stability and low melting temperature (Knothe, 2008). Similarly, palmitic, stearic, oleic and linoleic acid were the most common fatty acids contained in biodiesel (Knothe, 2008).

In a similar study, Chader et al. (2011) noted that the major fatty acid composition of *Chlorella sorokiniana* was a mixture of unsaturated fatty acids, such as oleic (18:1), linoleic (18:2) and linolenic acid (18:3) and trace amounts of saturated fatty acids, palmitic (16:0) and stearic (18:0). Biodiesel feedstock standards require high total fatty acid and low PUFA content (Doan and Obbard, 2012). For example, the International Biodiesel Standard for Vehicles (EN14214) requires the level of linolenic acid methyl ester (18:3n6, 18:3n3) and PUFA in final biodiesel fuel to be less than 12 and 1% (m/m), respectively. The present research is consistent with this requirement since linolenic acid was either not detectable or is lower than this range.

## *Properties of the oils and fatty acid methyl esters (biodiesel) from the four microalgae*

Properties of the oils from the four microalgae are presented in Table 4 with values not greatly differing from the FAME values of Table 5. The densities of the microalgae FAME are within the range of 0.867 to 0.882 g $ml^{-1}$, the viscosity values fell within 3.55 and 4.50, and flash points between 148 ± 0.22 and 165 ± 1.22°C. The range of iodine values ($10^{-4}$ Pa s), acid values (mg $KOHg^{-1}$)

**Table 4.** Properties of the oils from the four microalgae.

| Parameter | ***Oocystis* IA1 oil** | ***Chlorella* IA7 oil** | ***Chlorococcum* KA9 oil** | **B*otyrococcus* YA5 oil** |
|---|---|---|---|---|
| Density (at 15°C) ($gml^{-1}$) | 0.881 ± 0.01 | 0.878 ± 0.02 | 0.882 ± 0.04 | 0.877 ± 0.02 |
| Viscosity at 40°C | 3.85 ± 0.05 | 4.60 ± 0.09 | 4.50 ± 0.15 | 4.70 ± 0.04 |
| Flash point (°C) | 148 ± 0.22 | 155 ± 0.02 | 157 ± 0.11 | 165 ± 1.22 |
| Iodine value ($10^{-4}$ Pa s) | 120.0 ± 1.09 | 109.9 ± 1.01 | 118.5 ± 0.05 | 116.1 ± 0.92 |
| Acid value (Mg $KOHg^{-1}$) | 0.490 ± 0.05 | 0.388 ± 0.01 | 0.510 ± 0.06 | 0.533 ± 0.04 |
| Saponification value (Mg $KOHg^{-1}$) | 161 ± 1.02 | 154 ± 1.01 | 160.0 ± 0.92 | 161.5 ± 0.85 |

Results are means of triplicate tests.

**Table 5.** Properties of the biodiesel from the four microalgae in comparison with diesel fuel and standard biodiesel.

| Biodiesel properties | FAME *Oocystis* IA1 | FAME *Chlorella* IA7 | FAME *Chlorococcum* KA9 | FAME *Botyrococcus* YA5 | Diesel fuel | Standard biodiesel values | Test methods |
|---|---|---|---|---|---|---|---|
| Density (at 15°C) ($gml^{-1}$) | 0.867 ± 0.02 | 0.861 ± 0.04 | 0.872 ± 0.01 | 0.867 ± 0.02 | 0.838 | 0.860-0.900 | EN ISO 3675 |
| Viscosity at 40°C | 3.15 ± 0.12 | 4.10 ± 0.09 | 4.20 ± 0.15 | 4.15 ± 0.04 | 9 - 4.1 | 2.0 - 5.0 | EN ISO 3104 ASTMD445 |
| Flash point (°C) | 126 ± 1.20 | 125 ± 0.77 | 127 ± 0.18 | 125 ± 1.05 | 75 | 100 min | ISO 3679 ASTM D93 |
| Iodine value ($10^{-4}$ Pa s) | 110.0 ± 1.22 | 113.5 ± 0.55 | 112.4 ± 0.36 | 111.2 ± 1.08 | | 130 max | EN 14111 |
| Acid value (Mg $KOHg^{-1}$) | 0.210 ± 0.01 | 0.226 ± 0.03 | 0.190 ± 0.02 | 0.214 ± 0.01 | Max. 0.5 | 0.5 max. | ASTM D664, D974 |
| Saponification value (Mg $KOHg^{-1}$) | 182.2 ± 0.11 | 178.5 ± 0.98 | 180.0 ± 1.02 | 181.5 ± 1.11 | | | |

Results are means of triplicate tests.

and saponification value (mg $KOHg^{-1}$) were 109.9 ± 1.0 to 120.0 ± 1.09, 0.388 ± 0.01 to 0.533 ± 0.04 and 154 ± 1.01 to 161.5 ± 0.85, respectively, (Table 5). Although, the values varied slightly amongst the different fatty acid methyl esters, there was no significant difference between them. Biodiesel properties namely density (at 15°C), $gml^{-1}$; viscosity at 40°C; flash point (°C); iodine value ($10^{-4}$ Pa s); acid value (mg $KOHg^{-1}$); and saponification value (mg $KOHg^{-1}$) obtained in the present study were within the ASTM standard. The densities of the four-microalgal methyl esters were lower than their oil (Tables 4 and 5).

Lang et al. (2001) noted that the original crude oil, the refining steps and weather influence biodiesel density. Compared with the density of petro diesel, the four-microalgal methyl esters had higher densities. Conversely, the densities obtained in the present work are lower than those obtained for most vegetable oil methyl esters and from animal fats (Canakci and Sanli, 2008). In this study, even though the *Chlorococcum* KA9 methyl ester density was slightly higher than the densities from the other three, there was no significant difference among them.

The viscosities of the four microalgae methyl esters were significantly lower than their respective oils. The viscosities obtained in the present work are lower than those obtained for most vegetable oil and animal fat methyl ester (Canakci and Sanli, 2008). In this study, even though the *Oocystis* IA1 oil and methyl esters viscosities were apparently lower than those obtained from the remaining three, they were not statistically different from each other. The flash points of the oils were higher than their respective methyl esters for the four microalgae.

Compared to petro diesel, the values obtained in this study were higher than those reported of petro diesels. In the present study, the flash points of both the microalgal oils and their respective methyl esters were lower than that reported for vegetable oils and their respective biodiesels (Canakci and Sanli, 2008).

Iodine values obtained in this study for algal oil and their respective methyl esters were not significantly different from each other. This supported the observation previously made by Lang et al. (2001) that iodine values of biodiesel esters made from the same oil were similar. Acid values obtained in this study are statistically similar and there is no significant difference between the acid value of the methyl esters and their respective oils. The saponification value also agreed with that ASTM values for biodiesel.

## Conclusion

In this study, four strains of microalgae isolated from the arid environments of North East Nigeria have high potentials for biodiesel oil production. Isolation of native species that could produce biodiesel is important because the isolates are already adapted to the local environment. Characterization and further optimization of their culture conditions are currently under investigation.

## Conflict of interests

The authors did not declare any conflict of interest.

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