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

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African Journal of Biotechnology

*Review*

# Phytosynthesis of eco-friendly silver nanoparticles and biological applications – A novel concept in nanobiotechnology

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**Nano-biotechnology is an undoubtedly future generation technology which offers potential applications in multidisciplinary areas of science and technology. In the present day's production, stabilization and utilization of nanoparticles is the eliminatory division in modern science receiving immense attention of scientists engaged in different fields of research. A number of metal nanoparticles have been engineered so far; however among these, silver nanoparticles gain more attention because of their unique applications in distinctive fields of biology. This review presents an overview on phytosynthesis of silver nanoparticles; role of phytochemical constituents in reduction of silver nanoparticles, factors responsible for the synthesis of silver nanoparticles and their crucial role in control of size and shape etc. The biological applications of phyto-synthesized silver nanoparticles are given in brief which will direct a path for further biological studies in future to make the study more useful for human welfare and benefits.**

**Key words:** Phytosynthesis, silver nanoparticles, phytochemicals, biological activities.

## INTRODUCTION

In the present decade, nanotechnology has become one of the rapidly growing interdisciplinary areas of science and technology (Albrecht et al., 2006). The term nanotechnology was coined by Professor Norio Taniguchi of Tokyo Science University in the year 1974 to describe precision manufacturing of materials at the nanometer level (Taniguchi, 1974). The concept of nanotechnology was given by physicist Professor Richard P. Feynman in his lecture on there's plenty of room at the bottom (Feynman, 1959). This technology was found to be the

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platform for immeasurable technological innovations in 21st century. Research and development in the field of nanotechnology is growing at speed throughout the world. Development of new materials in the nanoscale including nanoparticles (NPs) is the major output expected in the study. NPs synthesis has received great attention from Chemists, Physicists, Biologists and Engineers who wish to use them for the development of a new generation of nanodevices (Shameli et al., 2012). NPs are clusters of atoms in the size range of 1 to 100 nm. Nano is a Greek word synonymous to dwarf meaning extremely small. NPs exhibit completely new or improved properties based on specific characteristics such as size, distribution and morphology (Mukunthan et al., 2011). In the recent past, nano material fabrication and their utilization is emerging as a cutting edge technology in different fields of human welfare, because of their distinctive characteristic features, such as, catalysis, electrical, optical, magnetic, chemical, mechanical properties etc.

Recently, a number of inorganic NPs have been synthesized by employing various procedures (Rao et al., 2012). NPs of copper, zinc, titanium, magnesium, plutonium, alginate, saponin, gold, silver and their alloys have come up. However, among these silver nano-particles (AgNPs), have wide range applications because of their remarkable physical and chemical properties obtained due to their large surface area to volume ratio and small particle size. AgNPs are excellent nano-materials providing a powerful platform in biomedical applications like bimolecular recognition, biosensing, drug delivery and molecular imaging (Chaloupka et al., 2010; Sironmani and Daniel, 2011). These AgNPs also have good antibacterial properties which are coming up as the current interest to researchers due to the growing microbial resistance against antibiotics and the development of resistant strains (Gong et al., 2007). These AgNPs are also used to alter and improve the pharmacokinetic and pharmacodynamic properties of various drugs to set them up for effective curing of various ailments (Mohanraj and Chen, 2006).

A number of approaches have been developed previously for the synthesis of AgNPs such as physical, chemical and biological (by using bacteria, lower forms of eukaryotes etc) methods (Gan and Li, 2012). Although these techniques have been well documented with greater prospects, they have certain limitations. During physical fabrication, metallic atoms are vaporized followed by condensation on various supports, in which the metallic atoms are rearranged and assembled as small cluster of metallic NPs (Egorova and Revina, 2004). The main advantage of the physical approach is that NPs with high purity and desired size can be selectively synthesized (Pol et al., 2002). A number of physical methods have been developed such as, sonochemistry (Gottesman et al., 2011), pyrolysis, physical vapor deposition, sol-gel, lithography, microwave irradiation (Yin et al., 2004), laser ablation (Tsuji et al., 2003), electro deposition, electrochemical (Rodrıguez-Sanchez et al., 2000;Yin et al., 2003), radiolysis (Thomas et al., 2005) methods etc. Most of these methods are expensive due to continuous consumption of energy to maintain the high pressure and temperature employed in NPs synthesis and requires highly sophisticated equipments.

Chemical approaches are the popular methods for synthesis of metallic NPs (Bönnemann and Richards, 2001). Several chemical reduction methods (Micelle, precipitation, chemical vapor deposition etc.) have been used to synthesize AgNPs from silver salts. Depending on the condition of reaction mixture, metal ions may favor either the process of nucleation or aggregation to form small metal clusters (Gan and Li, 2012). During the process of chemical synthesis, chemicals like sodium dodecyl sulphate, citrate of sodium, NaOH, KOH, $NH_4OH$, ethylene glycol etc. and some other toxic hazardous chemicals such as sodium borohydride and trisodium citrate were used as reducing agents; whereas,organic solvents (ethanol) (Kim, 2007) and some other non biodegradable compounds (synthetic polymers) were used as stabilizing agents. With the development of chemical methods, the concern for environmental contaminations is also highlighted as the chemical processes involved in the synthesis of NPs generate a large amount of hazards byproducts. The other main disadvantage of chemical synthesis is brought about during the process of synthesis;some toxic chemicals might be attached on the surface of NPs and may lead to adverse side effects during biological applications.

Although NPs are synthesized by using physical and chemical approaches, in contrary,biological systems came up as masters of ambient condition chemistry to synthesize metal NPs. The use of microorganisms in the synthesis of NPs was developed as relatively new and exciting area of research with considerable potential for development when compared to the above methods (Mohanpuria et al., 2008). Synthesis of AgNPs by using both prokaryotic and eukaryotic microorganisms such as bacteria, cyanobacteria, algae, fungi, actinomycetes protozoan's etc. were extensively investigated by several nano biologists. Biosynthesis of AgNPs (intracellular) by using the fungus *Verticillium* with size range in 25 ± 12 nm was reported by Mukherjee et al. (2001). The enzymatic synthesis of AgNPs by using *Fusarium oxysporum* was reported by Ahmad et al. *(*2003). Bhainsa and D'Souza (2006) investigated extracellular biosynthesis of AgNPs using *Aspergillus fumigatus.* Bioreduction and accumulation of AgNPs on the surface of cell wall within 72 h was reported in *Aspergillus flavus* (Vigneshwaran et al., 2007).

Synthesis of metal NPs (Au, Ag, Pd and Pt) with controlled size by using common cyanobacteria (*Anabaena*, *Calothrix* and *Leptolyngbya*) as bioreactors

was studied (Brayner et al., 2007). Lengke et al. (2007) reported the successful biosynthesis of AgNPs by using *Plectonema boryanum* UTEX 485 at 25 - 100°C for up to 28 days. Synthesis of Ag, Au and Au(core)/Ag(shell) NPs by using *Spirulina platensis* (single-cell protein) were achieved within 120 h at 37°C under controlled pH of 5.6 (Govindaraju et al., 2008). Biosynthesis of AgNPs using *Oscillatoria willei* NTDM01 was reported by Ali et al. (2011). The capability of protozoan's (*Leishmania* sp.) for synthesis of Ag and Au NPs was specifically reported by Ramezani et al. (2012). Biosynthesis of AgNPs by using culture supernatant of *Staphylococcus aureus* and their significant antibacterial effect against methicillin resistant bacteria such as *S. aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes*, was reported by Nanda and Saravanan (2009). Saifuddin et al. (2009) found that exposure of culture supernatanant of *Bacillus subtilis* with microwave irradiation to silver ions lead to the formation of AgNPs with size in the range of 5 - 60 nm. Reddy et al. (2010) reported that there is extensive extracellular bio-synthesis of AgNPs by using the same species *B. subtilis*.

Phyconanotechnology has also become one of the prominent fields of research in NPs synthesis (Narayanan and Sakthivel., 2011). Biosynthesis of AgNPs by using *Sargassum wightii* and their antibacterial effect on isolated pathogens of infected silk worms was reported (Govindaraju et al., 2009). The synthesis of AgNPs using the aqueous extract of *Gelidiella acerosa* as reducing agent and their significant antifungal effect was proved by Vivek et al. (2011). Synthesis of AgNPs from the extracts of *Sargassum plagiophyllum, Ulva reticulata* and *Enteromorpha compressa* and their characterization was reported by Dhanalakshmi et al. (2012). Prasad et al. (2013) reported the synthesis of AgNPs by using the extract of *Cystophora moniliformis* as both reducing and stabilizing agent. Even though the use of microorganisms (both prokaryotic and eukaryotic) in biosynthesis of AgNPs was well documented, they have certain drawbacks. It is very important to maintain controlled conditions for both microbial growth and reduction of NPs, standardization of medium composition for microbial growth and subsequent biosynthesis of AgNPs. Requirement of long duration time for growth and production of NPs is one of the main disadvantages while another disadvantage is that most of the microorganisms synthesize NPs intracellular which demands subsequent extraction, recovery steps that are more expensive.

The increasing interest in minimization of time, cost, waste etc, and implementation of sustainable process for the development of eco-friendly and simple techniques for the production of AgNPs lead to the development of photo biological approach. Photo biological approach refers to the synthesis of NPs by using plants or their extracts as reducing and stabilizing agents which is also known as green nanotechnology. Green nanotechnology is gaining much more attention due to its wide applications in pharmaceutical and biomedical fields.

Researchers are in search for biomaterials for the synthesis of AgNPs throughout the world because those synthesized through chemical and physical routes are not suitable for biomedical applications. The advantages of green nano science over traditional chemical and physical methods are that they do not use toxic chemicals during NPs synthesis, less expensive and easily scale up at the large scale. In the present review, phytosynthesis of AgNPs, factors affecting the size and morphology of AgNPs and their biological applications are discussed.

## PHYTOSYNTHESIS OF SILVER NANOPARTICLES IN HIGHER PLANTS

The development of reliable green process for the synthesis of AgNPs is an important aspect of current nanotechnology research (Sharma et al., 2012; Mittal et al., 2013). The role of higher plants in the synthesis of AgNPs is directly showing the relation between nanotechnology and Biotechnology. AgNPs were synthesized either by using the whole plants or plant pure compounds (glucose, starch, cellulose etc.), or by using plant dry mass or extracts etc (Song and Kim, 2008; Chandran et al., 2006; Song and Kim, 2009; Kasthuri et al., 2009). Initially the whole plants were used as biological factories for the synthesis of metallic NPs. The accumulation of AgNPs in the sprouts of alfalfa was investigated by Gardea-Torresdey et al. (2003). Haverkamp et al. (2007) reported the role of *Brassica juncea* germinating seeds in successful synthesis of Ag-Au-Cu alloy NPs; after which, Harris and Bali (2008) reported the use of whole plants to synthesize large quantities of AgNPs. They reported that it is feasible and found large uptake and reduction of silver ions and distribution of AgNPs within the cellular structures of *Brassica juncea* and *Medicago sativa*. However, the synthesis of AgNPs by using whole plants is cost effective and need to standardize the methods for further extraction and purification of NPs.

Followed by the use of whole plants for production of AgNPs, utilization of isolated pure compounds like glucose, starch, cellulose etc was extensively studied by several green nano Biologists. Park et al. (2011) explained clearly the role of polysaccharides and phytochemicals in Au and AgNPs synthesis. Raveendran et al. (2003) reported that β-D-glucose serves as the green reducing agent and starch serves as the stabilization agent for the synthesis of AgNPs. Latter *in situ* synthesis of noble metal (Ag, Au, Pt and Pd) NPs was carried out by using porous cellulose fibers (He et al., 2003). They found that the nanoporous structure and the high oxygen density of the cellulose fiber constitute an effective nano factory for *in situ* synthesis of metal NPs. Vigneshwaran et al. (2006) reported the production of stable AgNPs by using soluble starch as both the

reducing and stabilizing agents. They carried out this reaction in an autoclave at 15 psi, 121°C for 5 min. NPs thus prepared are found to be stable in aqueous solution over a period of three months at room temperature (25°C). The size of these NPs was found to be in the range of 10-34 nm.

Recently, the plant broth or extracts are being used directly for extracellular synthesis of AgNPs and it is extremely a cost effective method (Kumar and Yadav, 2009). Initially the use of *Pelargonium graveolens* leaf extract for the synthesis of metallic AgNPs were reported by Shankar et al. (2003). In continuation, the use of *Azadirachta indica* (Shankar et al., 2004), *Embelia ribes* (Ankamwar et al., 2005), *Aloe vera* (Chandran et al., 2006) and *Cinnamon camphora* (Huang et al., 2007) were investigated for synthesis of AgNPs. Later, from the past six to seven years, extensive study was conducted on many plant species such as *Cinnamomum zeylanicum* (Sathishkumar et al., 2009), *Capsicum annuum* (Li et al., 2007), *Helianthus annuus*, *Basella alba*, *Oryza sativa*, *Saccharum officinarum*, *Sorghum bicolor*, *Zea mays* (Leela and Vivekanandan, 2008), *Mentha piperata* (Parashar et al., 2009a), *Parthenium hysterophorus* (Parashar et al., 2009b), *Pinus desiflora*, *Diopyros kaki* , *Magnolia kobus* (Song and Kim, 2009), *Jatropha curcas* (Bar et al., 2009), *Gliricidia sepium* (Raut et al., 2009), *Bryophyllum*, *Cyperus, Hydrilla* (Jha et al., 2009) etc. (Table 1), to standardize the commercial synthesis of AgNPs. AgNPs were synthesized by using extracts from various parts in different plant species such as leaf, stem bark, roots, rhizomes, tubers, latex, flower, fruits, seeds etc. Rajasekharreddy et al. (2010) studied production of AgNPs by using leaf extracts from different plants (*Tridax procumbens*, *Jatropha curcas*, *Calotropis gigantea*, *Solanum melongena*, *Datura metel*, *Carica papaya* and *Citrus aurantium*) by sunlight exposure method. Production of biogenic AgNPs using *Boswellia ovalifoliolata* stem bark was reported by Ankanna et al. (2010). Biomimetic of AgNPs by using *Citrus limon* juice and theoretical prediction of particle size was studied by Prathna et al. (2011). Umadevi et al. (2012) investigated on AgNPs synthesis by using various concentrations of *Daucus carota* extract and Mukunthan and Balaji (2012) extensively studied the scale up process. Efficient production of AgNPs from papaya fruit extract was reported by Jain et al. (2009). Ghosh et al. (2012) reported that tuber extract mediated synthesis of AgNPs in *Dioscorea bulbifera*. Bankar et al. (2010) found that banana peel extract was used as a novel route for the synthesis of AgNPs. Green synthesis of AgNPs by using *Jatropha curcas* latex and seed extracts were reported by Bar et al. (2009a, b). Ahmad et al. (2012) synthesized AgNPs by using peels of *Punica granatum*. Fruit extracts of *Capsicum annuum* and *Dioscorea oppositifolia* were extensively investigated to synthesize AgNPs (Anal and Prasad, 2011; Maheswari et al., 2012a). Roopan et al. (2013) investigated low cost method to synthesize AgNPs by using *Cocos nucifera* coir.

## PLANT EXTRACTS AND THEIR CHEMICAL CONSTITUENTS

Plant mediated synthesis of AgNPs is regarded as a phytosynthesis or photo biological process in which the plant biomass itself is sufficient to reduce Ag ions into AgNPs. In this process,formation of NPs was proposed to occur through either the ionic or electrostatic interactions between the metal complexes and the functional groups of biomass source. Synthesis of AgNPs requires three key components; they are the solvent medium, reducing and stabilizing agents. In phytosynthesis of AgNPs the primary and secondary metabolites themselves can act as both reducing and stabilizing agents. Aqueous medium instead of organic solvents are used for green synthesis of AgNPs which is apparently regarded as more ecofriendly. Involvement of proteins, polyphenols and carbohydrates in the synthesis of metal NPs were reported by several biologists. All these constituents are present in plants and might be responsible for the synthesis of metal NPs. However, in plants, the involvement of such constituents in NPs synthesis needs experimental proof. Isolated quercetin (natural plant pigment) and polysaccharides have been used for AgNPs synthesis.

Park et al. (2011) explained in-depth the role of polysaccharides and phytochemicals in green synthesis of Au and AgNPs. The primary metabolites (biomolecules such as reducing sugars, proteins, peptides, amino acids etc.), present in plant extracts play a vital role in reducing and stabilizing of metallic silver into AgNPs. The ability of sugars as reducing agents for the synthesis of metallic NPs was reported by Panigrahi et al. (2004). Kwon et al. (2009) reported that the aldehyde group of succinoglycan sugar was oxidized to carboxyl group by nucleophilic addition of OH-, which reduced $Ag^{+}$ to Ag (0). Interaction of amino acids (as arginine, cysteine, lysine and methionine) with Ag ions was reported in earlier 1970's (Gruen, 1975). Several researchers reported that along with the bioreduction, proteins can act as stabilizing agents too. The role of peptides as bioreducing and biocapping agents were demonstrated by AgNPs synthesized by cyclic peptides in the latex of *Jatropha curcas* (Bar et al., 2009a) and targeted peptides enriched with proline and hydroxyl-containing amino acid residues (Naik et al., 2002). The effective role of proteins and enzymes in NPs synthesis was reported by Shankar et al. (2003). Proteins present in the *Nelumbo nucifera* leaf extract are essential for bioreduction of AgNPs (Santhoshkumar et al., 2010). Involvement of citric acid and some other bioorganics in the synthesis of AgNPs was reported by Prathna et al. (2011). Silver nano-particles are formed in natural rubber matrix via photo reduction of film cast from natural rubber latex containing

**Table 1.** List of species and their parts used for synthesis of silver nanoparticles and their biological applications.

| Species | Part used | Biological activity | Shape of nano particles | Size (nm) | Reference |
|---|---|---|---|---|---|
| *Acalypha indica* L. | Leaf | Antibacterial | - | 20 -30 | Krishnaraj et al., 2010 |
| *Achyranthes aspera* L. | Leaf | - | Nano triangle | - | Venkatesh et al., 2013 |
| | Leaf | Toxicity | Circular | 20-30 | Daniel et al., 2011 |
| *Acorus calamus* L. | Rhizome | Antimicrobial | - | - | Prashanth et al., 2011 |
| *Adhatoda vasica* Nees | Leaf | Antibacterial/anti proliferative | Spherical | 16.8 | Ranade et al., 2013 |
| | Arial parts | Antioxidant, Antidiabetic and Antimicrobial | Spherical to oval | 15-20 | Bandi and Yasundhara,2012 |
| *Aegle marmelos* (L.) Correa | Plant | Antimicrobial and cytotoxicity | Spherical | 0.9-1.2 | Lokina and Narayanan, 2011 |
| | Leaf | - | Spherical | 60 | Rao and Paria, 2013 |
| *Albizia adianthifolia (*Schum.) W. F. Wight. | Leaf | Anticancer | - | - | Govender et al., 2013 |
| | Leaves | Cytotoxicity | - | 4 - 35 | Gengan et al., 2013 |
| *Allium cepa* L. | Bulb | Antibacterial | Spherical | 33.67 | Saxena et al., 2010 |
| | Bulb | Antibacterial | - | 33.6 | Benjamin and Bharathwaj 2011 |
| *Allium sativum* L. | Bulb | Cytotoxicity | Spherical | 4 .4±1.5 | White II et al. 2012 |
| *Aloe vera* (L.) Burm. f. | Whole plant | - | Spherical | 15.2±4.2 | Chandran et al., 2006 |
| *Ammannia baccifera* L. | Aerial | Larvicidal | Spherical, triangle and hexagonal | 10-30 | Suman et al., 2013 |
| *Anacardium occidentale* L. | Leaf | - | Cubic | - | Sheny et al., 2011 |
| *Ananas comosus* Merrill | Leaf | - | Spherical | 12 | Ahmad and Sharma, 2012 |
| *Andrographis paniculata* Nees | Leaf | - | Cubic or hexagonal | 28 | Sulochana et al. 2012 |
| *Andropogon muricatus* Retz. | Root | - | - | - | Prashanth et al., 2011 |
| *Annona squamosa* L. | Leaf | Larvicidal | Spherical | 450 | Arjunan et al., 2012 |
| | Leaf | Antibacterial | Spherical | 28.47 | Jagtap and Bapat , 2012 |
| | Leaf | - | Cubic | 50-200 | Rajani et al., 2010 |
| *Arbutus unedo* L. | Leaf | - | Spherical | Up to 30 | Kouvaris et al., 2012 |
| *Argemone mexicana* L. | Leaf | Antibacterial | Cubic / Hexagonal | 20 | Singh et al., 2010 |
| | Leaf | - | - | - | Khandelwal et al., 2010 |
| *Astragalus gummifer* Labill. | Gum tragacanth | Antibacterial | Spherical | 13.1±1.0 | Kora and Arunachalam, 2012 |

**Table 1.** Contd.

| Species | Part used | Biological activity | Shape of nano particles | Size (nm) | Reference |
|---|---|---|---|---|---|
| | Leaf | Antimicrobial | Spherical | 43 | Gavhane et al., 2012 |
| | Leaf | - | Spherical | 50-100 | Shankar et al., 2004 |
| *Azadirachta indica* A.Juss | Leaf | - | Nearly spherical | Below 20 | Tripathy et al., 2010 |
| | Leaf | Antibacterial | - | - | Tripathi et al., 2009 |
| | Leaf | - | - | - | Anuradha et al., 2011 |
| *Baliospermum montanum* (Wild.) Muell-Arg | Leaf | Antibacterial and anticancer | - | 10 - 60 | Renugadevi et al., 2012 |
| *Basella alba* L. | Leaf | - | - | - | Leela and Vivekanandan, 2008 |
| *Berberis aristata* DC. | Wood | - | - | - | Prashanth et al., 2011 |
| | Stem bark | Antimicrobial | - | - | Savithramma et al., 2011 |
| *Boswellia ovalifoliolata* Bal. & Henry | Stem bark | - | Spherical | 30 - 40 | Ankanna et al., 2010 |
| | Leaves | Antimicrobial | - | - | Savithramma et al., 2012 |
| *Brassica chinensis* L. | Hypocotyls | - | - | - | Tan et al., 2010 |
| *Brassica juncea* Czern | *In vitro* cultures | - | - | - | Shekhawat and Arya, 2009 |
| *Brassica napus* L. | Somatic embryos | - | - | - | Tan et al., 2010 |
| *Brassica oleracea* L. | Leaf | - | - | - | Veeranna et al., 2013 |
| | Flower | Antibacterial | Spherical | 53.8 | Sridhara et al., 2013 |
| *Bryophyllum pinnatum* (Lam.) Oken. | Leaf | Antibacterial | Spherical | 70-90 | Baishya et al., 2012 |
| *Calotropis gigantea* L. | Leaf | - | Spherical | < 20 | Rajasekharreddy et al., 2010 |
| | Leaf | - | - | 11.8-83.7 | Sivakumar et al., 2011 |
| *Calotropis procera* (Aiton) W.T.Aiton | Flower | - | Cubic | 35 | Babu and Prabu, 2011 |
| | Leaf | - | Spheroidal and some are inprisms or rods | 20 | Begum et al., 2009 |
| *Camellia sinensis* (L.) Kuntze | Leaf | - | Anisotropic | 200 | Vilchis-Nestor et al., 2008 |
| | Leaf | - | Cubic | 20-60 | Nadagouda and Varma, 2008 |
| | Leaf | - | Cubic | 4 | Loo et al., 2012 |
| *Capscium annuum* L. | Fruit | - | - | - | Li et al., 2007 |
| | Fruit | - | Spherical | 2-6 | Jha and Prasad, 2011 |
| | Fruit | Antimicrobial | Cubic and hexagonal | 15 | Jain et al., 2009 |
| *Carica papaya* L. | Leaf | - | - | - | |
| | Callus | - | Spherical | 60 - 80 | |

**Table 1.** Contd.

| Species | Part used | Biological activity | Shape of nano particles | Size (nm) | Reference |
|---|---|---|---|---|---|
| *Cassia angustifolia* M. Vahl | Leaf | Antimicrobial | Spherical | 21.6 | Amaladhas et al., 2012 |
| *Cassia auriculata* L. | Leaf | - | Spherical | 20 - 40 | Udayasoorian et al., 2011 |
| | Flower | Antimicrobial | Spherical | 10-50 | Jobitha et al., 2012 |
| *Cassia fistula* L. | Leaf | | Nano wires | 40-60 | Lin et al., 2010 |
| *Catharanthus roseus* (L.) G. Don | Leaf | Antibacterial | Spherical | 48-67 | Mukunthan et al., 2011 |
| | Leaf | Antiplasmodial | Cubic | 35 - 65 | Ponarulselvam et al., 2012 |
| | Leaf | - | - | 45-70 | Kannan et al., 2011 |
| | Callus | Antimicrobial | - | - | Malabadi et al., 2012 |
| | *In vitro* derived plants | Antimicrobial | - | - | Malabadi et al., 2012 |
| | Dried leaves | Antimicrobial | Crystalline | 27±2 and 30±2 | Kotakadi et al., 2013 |
| *Celastrus paniculatus* Willd. | Seed | - | - | - | Prashanth et al., 2011 |
| *Centella asiatica* (L.) Urb. | Plant | Antimicrobial | - | 28.4 | Logeswari et al., 2012 |
| *Ceratonia siliqua* L. | Leaf | Antibacterial | Crystalline | 18 | Awwad et al., 2013 |
| *Chenopodium album L.* | Leaf | - | Quasi-spherical | 12 | Dwivedi and Gopal, 2010. |
| *Chromolaena odorata* (L.) King and Robinson | Leaf | - | Hexagonal | 40-60 | Geetha et al., 2012 |
| *Chrysopogon zizanioides* (L.) Roberty | Leaf | Antibacterial, antioxidant and cytotoxic | Cubic | 85-110 | Arunachalam and Annamalai, 2013 |
| *Cinnamomum camphora* (L.) J. Presl | Leaf | - | Spherical | 55-80 | Huang et al., 2007 |
| *Cinnamon zeylanicum* Nees. | Bark powder/extract | Antibacterial | Quasi-spherical and small rod-shaped | 31-40 | Sathishkumar et al., 2009 |
| *Cissus quadrangularis L.* | Stem | Antiphrastic | Spherical and oval | 42.46 | Santhoshkumar et al., 2012 |
| *Citrullus colocynthis* (L.) Schrad. | Leaf | Biomedical | Spherical | 31 | Satyavani et al., 2011 |
| | Calli | Antibacterial | Spherical | **75** | Satyavani et al., 2011 |
| *Citrus aurantium* L. | Leaf | - | - | - | Rajasekharreddy et al., 2010 |
| *Citrus limon* (L.) Burm.f. | Lime juice | - | spherical and spheroidal | 50 | Prathna et al., 2011 |
| *Citrus sinensis* (L.) Osbeck | Peel | Free radical scavenging, cytocompatible | Spherical | 6 | Konwarh et al., 2011 |
| *Citrus sinensis (L.) Osbeck* | Plant | Antimicrobial | - | 65 | Logeswari et al., 2012 |
| *Clerodendrum inerme (L.) Gaertn.* | Leaf | skin diseases | - | Different | Farooqui et al., 2010 |
| *Clitoria ternatea L.* | Callus and *in vitro* derived plants | Antimicrobial | - | - | Malabadi et al., 2012 |

**Table 1.** Contd.

| Species | Part used | Biological activity | Shape of nano particles | Size (nm) | Reference |
|---|---|---|---|---|---|
| *Cochlospermum gossypium DC.* | Gum | antibacterial | Spherical | 3 | Kora et al., 2010 |
| *Cocos nucifera* L. | coir | Larvicidal | Cubic | 23±2 | Roopan et al., 2013 |
| *Coffea Arabica* L. | Seed | - | Cubic | 20-60 | Nadagouda and Varma, 2008 |
| *Coleus aromaticus* Benth. | Leaf | Anti bacterial | Spherical | 44 | Vanaja and Annadurai., 2012 |
| *Commiphora wightii* (Arn.) Bhandari | Resin | - | - | - | Prashanth et al., 2011 |
| *Copaifera reticulata* Ducke | Oleoresin | Acaricidal | - | - | Fernandes and Freitas, 2007 |
| *Coriandrum sativum* L. | Fruit | - | - | - | Prashanth et al., 2011 |
| | Leaf | Nonlinear optics | Spherical | 26 | Sathyavathi et al., 2010 |
| *Cuminum cyminum* L. | Fruit | - | - | - | Prashanth et al., 2011 |
| *Curcuma longa* L. | Tubers | Bactericidal | Quasispherical, triangular and small rod-shaped | 71-80 | Sathishkumar et al., 2010 |
| *Cyamopsis tetragonaloba (*L.) Taub. | Leaf | - | - | - | Rajani et al., 2010 |
| *Cymbopogon citratus* (DC.) Stapf | Leaf | Antimicrobial | Spherical | 32 | Masurkar et al., 2011 |
| *Cynodon dactylon* (L.) Pers. | Plant | Antimicrobial and cytotoxicity | Cubic | - | Lokina and Stephen, 2011 |
| *Datura metel* L. | Leaf | - | - | - | Rajasekharreddy et al., 2010 |
| *Daucus carota L.* | Root | - | Spherical | 20 | Umadevi et al., 2012 |
| | Root | - | Spherical | 31-52 | Mukunthanand Balaji, 2012 |
| | Root | Antimicrobial and Cytotoxicity | - | - | Lokina and Narayanan, 2011 |
| *Desmodium triflorum* (L.) DC. | Plant | - | Spherical | 5-20 | Ahmad et al., 2011 |
| *Dioscorea bulbifera* L. | Tuber | Anti microbial | Nanorodsand triangles | 8-20 | Ghosh, et al., 2012 |
| *Dioscorea oppositifolia* L. | Fruit | Antimicrobial | Spherical | 14 | Maheswari et al., 2012 |
| *Diospyros kaki* Thunb. | Leaf | - | Au/AgNPs – cubic, AgNPs - spherical | Au/AgNPs - 50-500, AgNPS - 15-90 | Song and Kim, 2008 |
| *Drypetes roxburghii* (Wall.) Hurusawa. | Fruit | larvicidal | quasi-spherical | 26.6 | Haldar et al., 2013 |
| *Eclipta alba (L.) Hassk.* | Leaf | - | Spherical | 2-6 | Jha et al., 2009 |
| *Eclipta prostrata L.* | Leaf | Larvicidal | Spherical, Elongated | 55 - 60 | Rajakumar and Rahuman 2011 |
| *Elettaria cardamomom (L.) Maton* | Seed | Antimicrobial | Spherical | 40-70 | GnanaJobitha et al., 2012 |
| *Embelia ribes Burm.f.* | Fruit | - | - | - | Prashanth et al., 2011 |
| *Emblica officinalis L.* | Fruit | Transmetallation | - | 10-20 | Ankamwar et al., 2005 |

**Table 1.** Contd.

| Species | Part used | Biological activity | Shape of nano particles | Size (nm) | Reference |
|---|---|---|---|---|---|
| *Eucalyptus chapmaniana Cameron* | Leaf | Antimicrobial | Cubic | - | Sulaiman et al., 2013 |
| *Eucalyptus hybrida* | Leaf | - | Cubical | 50-150 | Dubey et al., 2009 |
| *Euphorbia hirta* L. | Leaf | Larvicidal | Spherical and cubic | 30 - 60 | Priyadarshini et al., 2012 |
| *Euphorbia hirta* L. | Leaf | antifungal | Spherical | 40-50 | Elumalai et al., 2010 |
| *Euphorbia milii* Des Moul. | Latex | Antimicrobial | -- | - | Patil et al., 2012 |
| *Euphorbia nivulia* Buch.Ham | Stem latex | Toxicity | Spherical, psudospherical | 10±2 | Valodkar et al., 2011 |
| *Euphorbia prostrata* Aiton | Leaf | Pesticidal | Rod | 25 - 80 average 52.4 | Zahir et al., 2012 |
| *Ficus benghalensis* L. | Leaf | Antibacterial | Spherical | 16-44 | Saxena et al., 2012 |
| *Ficus racemosa* L. | Bark | Larvicidal | - | - | Velayutham et al., 2013 |
| *Garcinia mangostana* L. | Leaf | Antimicrobial | Spherical | 35 | Veerasamy et al., 2011 |
| *Gliricidia specium* (Jacq.) Kunth ex Walp. | Leaf | Antibacterial | Spherical | 27 | Rajesh et al., 2009 |
| *Glycine max* (L.) Merr. | Leaf | - | Cubic | 25 - 100 | Vivekanandhan et al., 2009 |
| *Glycyrrhiza glabra* L. | Root/ Rhizome | - | - | - | Prashanth et al., 2011 |
| *Helianthus annuus* L. | Leaf | - | - | - | Leela and Vivekanandan, 2008 |
| *Hemidesmus indicus* R.Br. | Root | - | - | - | Prashanth et al., 2011 |
| *Hibiscus cannabinus* L. | Leaf | Antimicrobial | Spherical | 9 | Bindhu and Umadevi, 2013 |
| *Hibiscus rosa-sinensis* L. | Leaf | - | Different | ~13 | Philip, 2010 |
| *Holarrhena antidysenterica* Wall. | Seed | - | - | - | Prashanth et al., 2011 |
| *Hydrilla verticilata* (L.f.) Royle. | Whole plant | - | Spherical | 65.55 | Sable et al. 2012 |
| *Indigofera aspalathoides* DC. | Leaf | Wound healing | Square | 20-50 | Arunachalam et al., 2013 |
| *Jatropha curcas* L. | Leaf | - | Spherical | < 20 | Rajasekharreddy et al., 2010 |
| | Latex | - | - | 10 - 20 | Bar et al., 2009 |
| | Seed | - | Spherical | 15 - 50 | Bar et al., 2009 |
| | Latex | Antimicrobial | - | - | Patil et al., 2012 |
| *Jatropha gossypifolia* L. | Latex | Antimicrobial | - | - | Patil et al., 2012 |
| *Justicia genderussa* Burm.f. | Leaf | Cell viability | Spherical | 16 | Chinna and Prabha, 2012 |
| *Lactuca sativa* L. | Leaf | Antimicrobial | Spherical | 40-70 | Kanchana et al., 2011 |
| *Lantana camara* L. | Fruit | Antibacterial | Spherical | 12.55 | Sivakumar et al. 2012 |
| *Lawsonia inermis* L. | Leaf | Lousicidal | spherical | 59.52 | Marimuthu et al., 2012 |
| *Leonurus japonicas* Houtt. | Whole plant | Antibacterial | Spherical | 9.9-13.0 | Im et al. 2012 |
| *Leucas aspera* (Willd.) Link. | Bark | Antimicrobial | - | 29-45 | Antony et al., 2013 |
| *Lippia citriodora Kunth.* | Leaf | - | Spherical | 15-30 | Cruz et al., 2010 |
| *Macrotyloma uniflorum (Lam.) Verdc.* | Seed | - | Cubic | ~12 | Vidhu et al., 2011 |
| *Magnolia* spp. | Leaf | - | - | 15 - 500 | Song and Kim, 2009 |

**Table 1.** Contd.

| Species | Part used | Biological activity | Shape of nano particles | Size (nm) | Reference |
|---|---|---|---|---|---|
| *Mangifera indica L.* | Leaf | - | Spherical, triangular, hexagonal | 20 | Philip, 2011 |
| *Manilkara zapota* (L.) P.Royen | Leaf | Feeding deterrent activity | Spherical and oval | 70 - 140 | Kamaraj et al., 2012 |
| | Leaf | Acaricidal | Spherical and oval | 70 - 140 | Rajakumar and Rahuman, 2012 |
| *Mayaca fluviatilis* Aubl. | Fruit | - | - | - | Bunghez et al.,2010 |
| *Medicago sativa* L. | Sprouts | - | Wires | Different | Gardea-Torresdey et al., 2003 |
| *Melia azadirachta* L. | Bark | - | - | - | Prashanth et al., 2011 |
| | Leaf | Cytotoxic | Cubical and spherical | 78 | Sukirtha et al., 2012 |
| *Memecylon edule* Roxb. | Leaf | - | Square | 50 - 90 | Elavazhagan and Arunachalam, 2011 |
| *Mentha piperita* L. | Leaf | Antibacterial | Spherical | 90 | Mubarak Ali et al., 2011 |
| *Memecylon umbellatum* Burm.f. | Leaf | - | Spherical | 15-20 | Arunachalam et al., 2013 |
| *Mimosa pudica* L. | Leaf | Parasitic | Spherical | 25 - 60 | Marimuthu et al., 2011 |
| *Morinda pubescens* L. | Leaf | - | Spherical | 25-50 | Mary and Inbathamizh 2012 |
| *Morinda citrifolia* L. | Leaf | Antibacterial | Spherical | 27 | Sathishkumar et al., 2012 |
| | Root | Cytotoxicity | Spherical | 30-55 | Suman et al., 2013 |
| *Moringa oleifera* Lam. | Whole plant | - | - | - | Shivashankar and Sisodia, 2012 |
| *Morus alba* L. | Leaf | Antibacterial | Spherical | - | Wang et al., 2011 |
| | Leaf | Antibacterial | Cubic | 20-40 | Awwad and Salem, 2012 |
| *Murraya koenigii* (L.) Spreng. | Leaf | - | Cubodial | 20-35 | Suganya et al., 2013 |
| | Leaf | - | Spherical and ellipsoidal | 10 - 25 | Christensen et al., 2011 |
| *Murraya koenigii* (L.) Spreng. | Leaf | - | - | ~10 | Philip et al., 2011 |
| | Leaf | Larvicidal | Spherical and cubic | 20–35 | Suganya et al., 2013 |
| *Musa paradisiaca* L. | Leaf | Acaricidal and larvicidal | - | 50 - 150 | Jayaseelan et al., 2012 |
| | Peal | Antimicrobial | - | Up to 100 | Bankar et al., 2010 |
| *Myrtus communis* L. | Leaf | Antimicrobial | - | 60-100 | Sulaiman et al., 2013 |
| *Nigella sativa* L. | Seed | - | - | - | Prashanth et al., 2011 |
| *Nelumbo nucifera* Gaertn. | Leaf | Larvicidal | Triangular, pentagons, hexagonal | 25-80 | Santhoshkumar et al., 2011 |
| *Nerium oleander L.* | Leaf | Larvicidal | Spherical and cubic | 20-35 | Roni et al., 2013 |
| *Nicotiana tabaccum L.* | Leaf | - | Crystalline | 8 | Prasad et al., 2011 |
| *Ocimum* spp. | Leaf | | - | 3 - 20 | Mallikarjuna et al., 2011 |

**Table 1.** Contd.

| Species | Part used | Biological activity | Shape of nano particles | Size (nm) | Reference |
|---|---|---|---|---|---|
| *Ocimum canum Sim.* | Leaf | Acaricidal | Rod, cylindrical | 25 - 110 | Jayaseelan and Rahuman, 2012 |
| *Ocimum sanctum* L. | Leaf | Antibacterial | Spherical | 18 | Ramteke et al.,2013 |
| | Roots and stem | - | Spherical | 10±2 and 5±1.5 | Ahmad et al., 2010 |
| | Leaf | - | Triangle | 42 | Rao et al., 2013 |
| | Leaf | Antimicrobial | Spherical | 4 - 30 | Singhal et al., 2011 |
| | Leaf | Antigenotoxic | - | 50 | Vijaya et al., 2013 |
| | Leaf | Antibacterial and antifungal | Spherical | 50 | Rout et al., 2012 |
| *Ocimum tenuiflorum* L. | Plant | Antimicrobial | - | 28 | Logeswari et al., 2012 |
| | Leaf | Toxicity | Circular | 20 | Daniel et al., 2011 |
| | Leaf | Antibacterial | - | 25-40 | Patil et al., 2012 |
| | Leaf | Antibacterial | Spherical | 12 | Aniket et al., 2010 |
| *Oryza sativa* L. | Leaf | - | - | - | Leela and Vivekanandan , 2008 |
| *Paederia foetida* L | Leaf | Antimicrobial | Spherical | 4-15 | Mollick et al., 2012 |
| *Panicum virgatum* L. | Whole plant | - | Spherical, rod-like, triangular, pentagonal, hexagonal | 20 - 40 | Mason et al., 2012 |
| *Papaver somniferum* L. | Seed | - | Spherical | 3.2 - 7.6 | Vijayaraghavan et al., 2012 |
| *Parthenium hysterophorus* L. | Leaf | - | Various | Up to 90 | Sarkar et al., 2010 |
| | Leaf | - | - | - | Parashar et al., 2009 |
| *Pedilanthus tithymaloides* (L.) Poit. | Latex | Antimicrobial | - | | Patil et al., 2012 |
| | Leaf | Anti developmental | Spherical | 15-30 | Sundaravadivelan et al., 2013 |
| *Pelargonium graveolens* L. Her. | Leaf | - | Quasilinear | 16 - 40 | Shankar et al., 2003 |
| *Pennisetum glaucum* (L.) R. Br | Leaf | - | - | - | Rajani et al., 2010 |
| *Pergularia daemia* (Forssk.) Chiov. | Latex | Larvicidal | Spherical | 123.50 | Patil et al., 2012 |
| *Persimmon* spp. | Leaf | - | - | 15-500 | Song and Kim, 2009 |
| *Phyllanthus amarus* Schum. & Thonn | Phyllanthin | - | quasi-spherical | 30 | Kasthuri et al., 2009 |
| *Piper betle* L. | Leaf | - | Spherical | 3−37 | Mallikarjuna et al., 2012 |
| *Piper longum* L. | Leaf | Cytotoxicity | Spherical | 17.6-41 | Jacob et al., 2012 |
| *Piper nigrum L.* | Fruits | Free radical scavenging | Spherical | 40-60 | Mani et al., 2012 |
| | Leaf | - | Spherical | 5-60 | Mallikarjuna et al., 2012 |
| *Platanus orientalis L* | Leaf | - | - | - | Song and Kim, 2009 |

**Table 1.** Contd.

| Species | Part used | Biological activity | Shape of nano particles | Size (nm) | Reference |
|---|---|---|---|---|---|
| *Platanus* spp. | Leaf | - | - | 15-500 | Song and Kim, 2009 |
| *Plumbago rosea L.* | Root | - | - | - | Prashanth et al., 2011 |
| *Plumeria rubra* L. | Latex | Larvicidal | Spherical | 32 -220 | Patil et al. 2012 |
| *Polyalthia longifolia* Sonn. | Leaf | Antibacterial | Spherical | Various | Kaviya et al., 2011 |
| | Leaves | - | Spherical | 57.53 | Prasad et al., 2012 |
| *Pongamia pinnata* (L) Pierre | Leaf | Antibacterial | Spherical | 38 | Rajesh et al., 2010 |
| *Prosopis juliflora* DC. | Leaf | Antimicrobial | Triangles, tetragons, pentagons and hexagons | 11 -19 | Raja et al.,2012 |
| *Psoralea corylifolia* L. | Resin | - | - | - | Prashanth et al., 2011 |
| *Pulicaria glutinosa* (Boiss.) Jaub. & Spach | Aerial parts | - | Spherical | 40-60 | Khan et al., 2013 |
| *Punica granatum* L. | Peel | - | Spherical | 21 | Solgi and Taghizadeh, 2012 |
| | Peels | - | Spherical | 5 ±1.5 | Ahmad et al., 2012 |
| | Peels | Catalytic | Spherical | 30 | Edison and Sethuraman, 2013 |
| *Rhizophora mucronata* Lam. | Leaf buds | Antibacterial | - | 4 - 26 | Umashankari et al., 2012 |
| *Rosa damascena* Mill. | petals | - | Spherical | 21 | Solgi and Taghizadeh, 2012 |
| | Flower | Electro chemistry | - | 21 | Ghoreishi et al., 2011 |
| *Rosa rugosa* Thunb. | Leaf | - | Spherical | 12 | Dubey et al., 2010 |
| *Rosmarinus officinals* L. | Leaf | Antimicrobial and Cytotoxicity | Cubic | 60 | Sulaiman et al., 2013 |
| *Saccharum officinarum* L. | Leaf | - | - | - | Leela and Vivekanandan , 2008 |
| *Sesuvium portulacastrum* L | Callus and leaf | Antimicrobial | Spherical | 5-20 | Nabikhan et al., 2010 |
| *Shorea tumbuggaia* Roxb. | Leaf | Antimicrobial | - | - | Savithramma et al., 2012 |
| | Stem bark | Antimicrobial | - | - | Savithramma et al., 2011 |
| *Shorea robusta* Roth. | Stem bark | Antimicrobial | Spherical | - | Savithramma et al., 2011 |
| *Smilax china* L. | Root | - | - | - | Prashanth et al., 2011 |
| *Solanum lycopersicum* L. | Fruit | - | Spherical | 10 | Umadevi et al., 2013 |
| *Solanum melongena* L. | Leaf | - | - | - | Rajasekharreddy et al., 2010 |
| *Solanum torvum Sw.* | Leaves | Antimicrobial | Spherical | 14 | Govindaraju et al., 2010 |
| *Solanum trilobatum L.* | Plant | Antimicrobial | - | 22.3 | Logeswari et al., 2012 |
| | Leaf | Antidandruff | Cubic and Hexagonal | 15-20 | Pant et al., 2012 |

**Table 1.** Contd.

| Species | Part used | Biological activity | Shape of nano particles | Size (nm) | Reference |
|---|---|---|---|---|---|
| *Solanum xanthocarpum L.* | Berry | Antimicrobial and uriase inhibitory activity | Spherical | 10 | Amin et al., 2012 |
| *Sorbus aucuparia* L. | Leaf | - | Spherical, triangular, hexagonal | 16 | Dubey et al., 2010 |
| *Sorghum* SPP. | Bran | - | Spherical | ~10 | Njagi et al., 2011 |
| *Sorghum bicolor* (L.) Moench | Leaf | - | - | - | Leela and Vivekanandan, 2008 |
| *Sorghum vulgare* Pers. | Leaf | - | - | - | Rajani et al., 2010 |
| *Spinacia oleracea* L. | Leaf | Antimicrobial | Different | 40-70 | Kanchana et al., 2011 |
| *Suaeda monoica* Forssk. ex J. Gmelin | Leaf | Cell line toxicity | Spherical | 31 | Satyavani et al., 2012 |
| *Svensonia hyderabadensis* (Walp.) Mold | Leaf | Antimicrobial | - | - | Savithramma et al., 2012 |
| | Leaf | Antimicrobial | Spherical | 45 | Rao and Savithramma , 2011 |
| | Leaf | Antimicrobial | - | - | Savithramma et al., 2011 |
| | Stem | Antimicrobial | - | - | Rao and Savithramma, 2012 |
| *Swietenia mahogany* (L) Jacq. | Leaf | - | Spherical | 20-50 | Mondal et al. 2011 |
| *Syzygium cumini* (L.) Skeels | Leaf, seed | - | Spherical | LE-30, leaf water fraction-29, SE-92, seed water fraction -73 | Kumar et al., 2010 |
| | Plant | Antimicrobial | - | 26.5 | Logeswari et al., 2012 |
| *Tanacetum vulgare* L. | Fruit | - | Triangular, spherical and hexagonal | 10–40 | Dubey et al., 2010 |
| *Terminalia chebula* Retz. | Fruit | antimicrobial | Spherical | - | Kumar et al., 2012a |
| *Thespesia populnea* (L.) Sol. ex Correa | Leaves | Antimicrobial | - | - | Savithramma et al., 2012 |
| *Tinospora cordifolia* Miers | Leaf | Pediculicidal and Larvicidal | - | 55 - 80 | Jayaseelan et al., 2011 |
| *Trachyspermum ammi* Sprague | Seed | - | Triangular | 87 - 998 | Vijayaraghavan et al., 2012 |
| *Trianthema decandra* L. | Roots | Antimicrobial | Cubic | 15 | Geethalakshmi and Sarada, 2010 |
| *Tribulus terrestris* L. | Plant | Antimicrobial | Spherical | 16-28 | Gopinath et al., 2012 |
| *Tridax procumbens* L. | Leaf | - | Spherical | <20 | Rajasekharreddy et al., 2010 |
| *Trigonella foenum-graecum* L. | Seed | - | - | - | Prashanth et al., 2011 |
| *Triticum aestivum* L. | Seedlings | Peroxide Catalytic Activity and Toxicology | Polydispersed spherical | 10 | Waghmode et al., 2013 |
| *Triphala ingredients* | Fruit mixture | - | Spherical | 59 | Gavhane et al., 2012 |
| *Turnera ulmifolia L.* | Leaf | Antimicrobial | - | - | Shekhawat et al., 2012 |
| *Vigna radiate (L.) Wilczek.* | Leaf | Antibacterial | - | - | Rajani et al., 2010 |

**Table 1.** Contd.

| Species | Part used | Biological activity | Shape of nano particles | Size (nm) | Reference |
|---|---|---|---|---|---|
| *Vinca rosea* L. | Leaf | Antimicrobial | - | - | Savithramma et al., 2012 |
| | Leaf | Larvicidal | Spherical | 25- 47 | Subarani et al., 2013 |
| *Vitex negundo* L. | Leaf | Antibacterial | Cubic | 10-30 | Zargar et al., 2011 |
| *Wrightia tinctoria* (Roxb.) R.Br. | Leaf | - | - | 19-68 | Bharani et al., 2012 |
| *Zea mays* L. | Leaf | - | - | - | Rajani et al., 2010 |
| | Leaf | - | - | - | Leela and Vivekanandan , 2008 |
| *Zingiber offcinale* Roscoe. | Rhizome | - | Spherical | 6-20 | Kumar et al., 2012b |

silver salt was reported by Bakar et al. (2007).

Several active principles (secondary metabolites) such as flavonoids, terpenoids and alkaloids were suggested to be involved as either reducing or stabilizing agents during the formation of NPs. All these constituents are present in plants and might be responsible for the synthesis of metal NPs. However, in plants, the involvement of such constituents in NPs synthesis needs experimental proof. Isolated quercetin (natural plant pigment) has been used for AgNPs synthesis (Egorova and Revina, 2000; Kundu et al., 2004). Shankar et al. (2003) reported that terpenoids in geranium leaf may be responsible for the synthesis of AgNPs.

Shankar et al. (2004) reported that the flavonoids and terpenoids present in the leaf broth of *A. indica* are responsible for the production of stable Ag, Au and bimetallic Ag-Au NPs. *Cinnamomum zeylanium* bark is rich in terpenoids, including linalool, eugenol, and methyl chavicol, which contribute to its distinct aroma. It was suggested by Singh et al. (2010) that eugenol plays a critical role in bioreduction of AgNPs. Furthermore, the effective role of polyphenols and caffeine present in the coffee and tea extract in the synthesis of AgNPs were reported by Nadagouda and Varma (2008). In the case of hydrophytes, compounds such as catechol, potocatecheuic acid along with other phytochemicals in *Hydrilla* have been reported to liberate reactive hydrogen, which contribute to the reduction of AgNPs (Jha et al.,2009). In the present days, many of the researchers are investigating the phytochemicals that are involved in the bio reduction and stabilization of AgNPs and the possible mechanisms that are involved in reduction of AgNPs by using active principles (Philip, 2010; Njagi et al., 2011). In *Ananas comosus*, different types of antioxidants present in the juice synergistically reduce the Ag metal ions, as each antioxidant is unique in terms of its structure and antioxidant function (Ahmad and Sharma, 2012).

## FACTORS AFFECTING THE SIZE AND MORPHOLOGY OF Ag NANOPARTICLES

The intrinsic properties of metal NPs are mainly determined by its size, shape, composition, crystallinity and structure. Normally the optoelectronic and physiochemical properties of nanoscale matter strongly depend on particle size, whereas the particle shape contributes significantly to modulate their electronic properties. Green methods generally lead to the formation of crystalline NPs with sizes ranging in between 1 to 100 nm and with a wide variety of shapes (spheres, rods, prisms, plates, needles, leafs or dendrites).

The desired size and shape of the AgNPs can be achieved by controlling the process parameters (Chan and Don, 2013), such as the nature of plant extract and the relative concentrations of the extract and metal salts, pH, temperature, reaction time etc. The stability of produced NPs can in some cases change after a few days or the NPs can remain stable over longer periods. AgNPs were rapidly synthesized using the leaf extract of *Mimosa pudica* and the formation of NPs was observed within 6 h (Marimuthu et al., 2011). Song and Kim (2009) reported 90% formation of AgNPs within 11 min by using leaf broth of *Magnolia* at 95°C. Synthesis of AgNPs was started within 15 min and the reduction reaction was completed within 2 h by using the extract of *Panicum virgatum* (Mason et al., 2012). Sulaiman et al. (2013a) reported that when 10 ml of *Rosmarinus officinalis* extract was mixed with

90 ml of 2 mM $AgNO_3$ and heated at 70°C for 3 min, it resulted in rapid synthesis of AgNPs. Similarly, 10 ml of leaf extract of *Eucalyptus chapmaniana* mixed with 90 ml of 0.01 or 0.02 mM aqueous $AgNO_3$ and exposed for 1 h to sun light leads to synthesis of AgNPs (Sulaiman et al., 2013b). Temperature plays an important role in reduction of $AgNO_3$ to stable AgNPs. The effective role of temperature in NPs synthesis was well explained by Panda et al. (2011) and they found that the optimum colour intensity was achieved within 14 min at 95°C; whereas, the same reaction required 12 h at 25°C temperature. Krishnasamy et al. (2012) reported that complete reduction of silver ions observed after 48 h at 30°C under shaking condition while *Indigofera aspalathoides* leaf extract was used as reducing agent. Similar type of report was provided while *Cassia auriculata* leaf extract was used as reducing agent (Udayasoorian et al., 2011). The effect of different process parameters like the reluctant concentration, mixing ratio of the reactants etc. was studied (Prathna et al., 2011).

In this, $10^{-2}$ M $AgNO_3$ solution was interacted for 4 h with lemon juice in the ratio of 1:4 and formed AgNPs with size below 50 nm which are spherical or spheroid in shape. Christensen et al. (2011) studied the biological synthesis of AgNPs using *Murraya koenigii* leaf extract and the effect of broth concentration on reduction mechanism and particle size. Huang et al. (2011) reported that increasing the $AgNO_3$ concentration at 30 or 65°**C** increased the mean size and the size distribution of the AgNPs. Singhal et al. (2011) reported that *Ocimum sanctum* leaf extract can reduce $AgNO_3$ into AgNPs within 8 min of reaction time. When the *Mentha piperita* extract was subjected to $AgNO_3$,the biosynthesis reaction was started within few minutes (Mubarakali et al., 2011). AgNPs with short chain-shaped structures were produced by using *Cassia fistula* leaf extract (Lin et al., 2010). They observed that by prolonging the reaction time, the newly formed Ag atoms deposited onto the concave regions of the connected NPs through capillary phenomenon, leading to the formation of long nanorods (Lin et al., 2010). Vivekanandhan et al. (2009) studied the effect of different *Glycine max* varities leaf extracts on bioreduction of AgNPs. Ramteke et al. (2013) reported that when tulasi extract was used as reducing agent, more than 90% of the reaction was completed within one hour of the reaction time. It was found that AgNPs synthesized by *Medicago sativa* seeds formed aggregation of hexagonal and well-defined shaped NPs without signs of fusion. This could be attributed to the strong interaction between the chemically bound capping agents which counteracted the tendency of the NPs to aggregate (Lukman et al., 2011). Optimization studies revealed that the maximum rate of synthesis could be achieved with 0.7 mM $AgNO_3$ solution at 50°C in 5 h (Ghosh et al., 2012). The variation of particle size with the reaction temperature and reaction time has been reported (Sarkar et al., 2010).

## PHARMACOLOGICAL APPLICATIONS

Silver products have long been known to have strong inhibitory and bactericidal effects, as well as broad spectrum of antimicrobial activities which has been used for centuries to prevent and or treat various infections. Recent research regarding green synthesized AgNPs revealed that they have wide spread biological applications like free radical scavenging, biocompatibility, antimicrobial effects etc. (Konwarh et al., 2011).

## ANTIBACTERIAL STUDIES

The AgNPs show efficient antimicrobial property compared to other NPs due to their extremely large surface area, which provides better contact with microorganisms. Even though the exact mechanism involved in bactericidal activity of nanoscaled silver on bacteria was not fully understandable; the three most common mechanisms of toxicity were well explained by Jones and Hoek (2010).

Due to their nano size, AgNPs get attached to the cell membrane and penetrate easily into the bacteria. The bacterial membrane contains sulfur-containing proteins; the AgNPs interact with these proteins and diffuses into the cell. The NPs preferably attack the respiratory chain, cell division finally leading to cell death. The NPs release silver ions in the bacterial cells, which enhance their bactericidal activity. The NPs smaller than 10 nm interact with bacteria and produce electronic effects, which enhance the reactivity of NPs.

The bactericidal effect of the nano crystalline AgNPs was tested against *Escherichia coli* strain (BL 21) (Satishkumar et al., 2009). They found that different tested concentrations (2, 5, 10, 25 and 50 mg/L) exhibited different inhibition percentages (10.9, 32.4, 55.8, 82 and 98.8%), respectively. The calculated $EC_{50}$ value was 11 ± 1.72 mg/L and minimum inhibitory concentration (MIC), was found to be 50 mg/L. Strains with the same species were reported to possess different inhibitory effects when the same concentration of AgNPs were used (Geethalakshmi and Sarada, 2010). AgNPs exhibited significant antibacterial activity against *E. coli* and *Pseudomonas aeruginosa* showing clear inhibition zone at a concentration of 50 ppm (Jain et al., 2009). Antimicrobial assay of biosynthesized AgNPs against both Gram-negative (*E. coli*) and positive (*S. aureus*) microorganisms at different concentrations was studied (Singhal et al., 2011) and they revealed a strong dose-dependent antimicrobial activity against both the tested microorganisms. It was seen that, as the concentration of biosynthesized NPs were increased, microbial growth decreases in both the cases. Biosynthesized AgNPs were found to exhibit more antimicrobial activity on Gram-negative microorganism than Gram-positive ones. Mubarakali et al. (2011) found similar type of results while

studying antimicrobial activity of biosynthesized AgNPs and they reported that it may be due to the variation in the cell wall composition between Gram positive and Gram negative bacteria. Rajesh et al. (2010) reported that the AgNPs synthesized using dried leaves of *Pongamia pinnata* were effective against different strains of bacteria such as *E. coli* (ATCC 8739), *S. aureus* (ATCC 6538p), *P. aeruginosa* (ATCC 9027) and *Klebsiella pneumoniae* (clinical isolate). AgNPs with considerable growth inhibition of two of the well known pathogenic bacteria with zone of inhibition 11 mm (*E.coli*) and 10 mm (*S. aureus*), respectively were reported by Ramteke et al. (2013).

The inhibitory percentage of the AgNPs against *E*. *coli* and *S*. *aureus* at different concentrations shows that the higher the concentrations of NPs,the higher the inhibitory effect. The minimum inhibitory concentration for *E. coli* was 1.4 ppm; whereas, for *S. aureus*, it was 5.4 ppm (Huang et al., 2011). AgNPs synthesized by using aqueous extract of *Alium cepa* showed significant antibacterial activity (Benjamin and Bharathwaj, 2011). Kulkarni et al. (2011) reported that the AgNPs showed significant antimicrobial activity on four clinically isolated pathogens with zone of inhibitions on *P. aeruginosa* (12 mM), *E. coli* (11 mM), *Bacillus subtilis* (9 mM) and *K. pneumoniae*(8 mM), respectively. They found that with increasing the concentrations of AgNPs, there is a gradual reduction in bacterial growth of *E. coli*. The AgNPs were found to possess potent antibacterial activity against both Gram negative and Gram positive bacteria (Ghosh et al., 2012). They found that beta lactam (piperacillin) and macrolide (erythromycin) antibiotics showed a 3.6-fold and 3-fold increase, respectively in combination with AgNPs selectivity against multidrug-resistant *Acinetobacter baumannii.* Notable synergy was seen between AgNPs and chloramphenicol/vancomycin against *P. aeruginosa* and was supported by 4.9-fold and 4.2-fold increases in zone diameter, respectively. Similarly, a maximum 11.8-fold increase in zone diameter of streptomycin when combined with AgNPs against *E. coli* was found. This report provides a strong evidence for the synergistic action of a combination of antibiotics and AgNPs against multidrug resistant bacteria.

The AgNPs synthesized by using *Argemone mexicana* showed significant antibacterial activity towards *E. coli* and *Pseudomonas syringae* at a concentration range of 30 ppm (Singh et al., 2010). Synthesis of plant-mediated AgNPs using various medicinal plant extracts and evaluation of their antimicrobial activities was studied by Prashanth et al. (2011). The antimicrobial activities of colloidal silver particles are influenced by the dimensions of the particles (Kaviya et al., 2011). They found that smaller particles lead to the greater antimicrobial effects. Finally, they concluded that the AgNPs synthesized at 60°C showed significant antibacterial effect when compared to those synthesized at 25°C because of their smaller size. Synthesis of AgNPs from stem bark extracts of *Boswellia, Shorea* and leaf extract of *Svensonia* were studied (Savithramma et al., 2011). They noticed that AgNPs synthesized from bark extracts of *Boswellia ovalifoliolata* and *Shorea tumbuggaia* showed toxicity towards *Klebsiella* and *Pseudomonas* species, respectively;whereas, the growth of *Pseudomonas* alone was inhibited maximumly by the AgNPs synthesized from leaf extract of *Svensonia hyderobadensis*. The antimicrobial activity of AgNPs from neem and triphala was evaluated against multiple drug resistant hospital isolates of *E. coli*, *K. pnuemoniae* and *S. typhi*. They showed clear zone of inhibition against all the tested microorganisms. Zone of inhibition was found to be in the range of 11-14 mm for neem AgNPs whereas 10 - 14 nm for Triphala (Gavhan et al., 2012). Renugadevi et al. (2012) reported that the AgNPs showed significant antibacterial activity against all tested microorganisms. The maximum activity was found against *E. coli* followed by *S. aureus* and *Salmonella typhi*;whereas, least activity was found against *Vibrio cholerae, K. pneumoniae* and *Bacillus subtilis.* Green synthesis of AgNPs using *Bryophyllum pinnatum* and *Cassia angustifolia* and their promising antibacterial activity against *E. coli* and *S. aureus* were reported (Baishya et al., 2012;Amaladhas et al., 2012). Veeranna et al. (2013) reported that cabbage AgNPs showed significant antibacterial activity towards *S. aureus* with zone of inhibition of 13.4 mm.

The application of AgNPs as an antimicrobial agent was investigated by Sulaiman et al. (2013) against human pathogens. They found that, the antimicrobial effect was dose-dependent, and were more pronounced against Gram-positive bacteria than Gram-negative bacteria. Efficient antimicrobial activity with maximum zone of inhibition towards *E. coli* (14 mm), *S. aureus* (13 mm) and with minimum zone of inhibition towards *B. cereus* (7 mm); *K. pneumoniae* (7 mm) and *C. krusei* (6 mm) was reported by Prasad et al. (2012). The efficient antibacterial activity was noticed with stabilized AgNPs against all the tested pathogens (Malabadi et al., 2012) such as *B. subtilis, S. aureus*, *E. coli* and *K. pneumoniae*. Biosynthesis of AgNPs using *Ulva fasciata* extract and its significant antibacterial activity against *Xanthomonas campestris*pv. *Malvacearum* was reported by Rajesh et al. (2012). The maximum bactericidal effect of AgNPs of *Turnera ulmifolia* was against *E*. *coli*, *P*. *aeroginosa* followed by *S.aureus* and *Enterococcus faecalis* (Shekhawat et al., 2012). Antimicrobial effects of AgNPs against marine aquatic pathogens such as *Pseudomonas florescens*, *Proteus* spp. and *Flavobacterium* spp. was studied (Umashankari et al., 2012). They observed that the antimicrobial effect varies according to the species.

The maximum zone was recorded as 16, 14 and 14 mm for *Pseudomonas florescens*, *Proteus* spp., and *Flavobacterium* spp. respectively at 75 µg/µl concentration. *In vitro* anti-*Helicobacter pylori* activity of synthesized AgNPs was tested against 34 clinical isolates and two reference strains of *Helicobacter pylori* (Amin

et al., 2012).They noticed that typical AgNPs effectively inhibited the growth of *H. pylori*, indicating that the AgNPs have stronger anti-*H. pylori* activity. Finally they stated that AgNPs under study were found to be equally efficient against the antibiotic-resistant and antibiotic-susceptible strains of *H. pylori*. Rao and Savithramma (2012) found that AgNPs exhibited maximum antibacterial effect towards *Pseudomonas* spp. with zone of inhibition 18 mm among all the bacterial species tested. Sulaiman et al. (2013) has reported that 2 mM AgNPs induced 25 mm clear inhibitory zone against *S. aureus* and *S. pneumoniae* followed by 22 mm for Gram-negative bacteria such as *E. coli*, *K. pneumonia*, *P. aeruginosa* and *Proteus vulgaris* respectively.

## ANTIFUNGAL ACTIVITY

The synthesized AgNPs were generally found to be effective as antimicrobial agents against some of the important human pathogens (Maheswari et al., 2012). AgNPs of *Argemone mexicana* showed significant antifungal activity towards *Aspergillus flavus* at a concentration range of 30 ppm (Singh et al., 2010). Elumalai et al. (2010) reported that AgNPs synthesis by green route was found highly toxic against 7 clinically isolated fungal species at a concentration of 50 µg/µl; the maximum activity was observed against *Candida albicans, C. kefyr, A. niger* followed by intermediated activity towards *C. tropicalis, C. krusei, A. flavus* and *A. fumigatus*. AgNPs synthesized from bark extracts of *Boswellia ovalifoliolata* and *Shorea tumbuggaia* showed toxicity towards *Aspergillus* and *Fusarium* species, respectively; whereas, the growth of *Rhizopus* species were inhibited maximumly by AgNPs synthesized from leaf extract of *Svensonia hyderobadensis* (Savithramma et al., 2011). The antifungal activity of AgNPs from neem and Triphala was checked against hospital isolate (*C. albicans*) and found that the zone of inhibition was in the range of 15 and 16 mm, respectively (Gavhan et al., 2012). Rao and Savithramma (2012) reported that among all the tested fungi, the AgNPs showed higher activity towards *Rhizopus* with maximum zone of inhibition 14 mm. The significant antifungal activity of AgNPs with maximum inhibition towards C*. albicans* was reported by Sulaiman et al. (2013).

## ANTIPLASMODIAL STUDIES

The antiplasmodial activity of AgNPs synthesized from *Catharanthus roseus* were tested against malarial parasites. Lowest parasitemia inhibition (20.0%) was found in parasites at 25 g/mL concentration of AgNPs. The parasitemia inhibitory concentration values vary with the concentration of AgNPs (20.0, 41.7, 60.0 and 75.0% for 25, 50, 75 and 100 g/ml), respectively (Ponarulselvam et al., 2012).

## CYTOTOXICITY AND GENOME TOXICITY

Genotoxicity of AgNPs was assessed by using well-established *Allium cepa* assay system with biomarkers including the generation of reactive oxygen species (ROS: $O_2$ and $H_2O_2$), cell death, mitotic index, micronucleus, mitotic aberrations; and DNA damage (Panda et al., 2011). They also used other chemical forms of silver such as $Ag^+$ ion, colloidal AgCl and AgNPs at doses 0 - 80 mg/L to compare with biogenic AgNPs. They found that commercial AgNPs and biogenic AgNPs exhibited similar biological effects. Both NPs (commercial and biogenic) causes lesser cytotoxicity and greater genotoxicity. It has been reported that the potential to induce cell death in root tissue of *A.cepa* of different forms of silver follows the order: silver ions > colloidal AgCl > commercial AgNPs > biogenic AgNPs. Cell death and DNA-damage induced by biogenic AgNPs were prevented by tiron and dimethyl thiourea that scavenge $O_2$ and $H_2O_2$, respectively. In another report, the toxicity of biogenic silver nanoparticles produced by *Alternaria alternata*, capped with protein, and in sizes of 25 - 45 nm, was evaluated for DNA damage in human lymphocytes using comet assay. The trypan blue dye exclusion method showed no significant changes in cellular viability on exposed cells compared with untreated control cells (up to 400 mg/ml). The *in vitro* treatment of lymphocytes using comet assay for DNA damage evaluation showed that, up to 50 mg/ml of nanoparticles, no DNA damage was observed. However, over 100 mg/ml with the increase in the concentration of silver nanoparticles, an increase in DNA damage was observed up to300 mg/ml, as represented in terms of percentage of DNA in the tail and olive tail moment test. The values of comet parameters were ~ 5-fold higher in the positive control (100 mmol/L methyl methanesulphonate) compared with the lowest treatment dose (Sarkar et al., 2011).

Sulaiman et al. (2013) employed a time and dose dependent approach to evaluate the toxicity of the AgNPs on human acute promyelocytic leukemia (HL-60). They found that the viability of HL-60 cells considerably decreased with increasing doses and time of incubation. The mortality data obtained allow them to predict their potential not only because of the cytotoxic effect, but also in terms of the potential for tumor reduction. They stated that cytotoxic effects of AgNPs may be due to the result of active physicochemical interaction of NPs with the functional groups of intracellular proteins, as well as with the nitrogen bases and phosphate groups in DNA. *In vitro* cytotoxic effects of AgNPs were screened against HL-60 cell line and viability of tumor cells was confirmed using MTT assay (Sulaiman et al., 2013). They found that after six hours of treatment, AgNPs at 2 mM level decreased the viability of HL-60 cells up to 44% and they also noticed that longer exposures resulted in additional toxicity to the cells that is, 80% after 24 h of incubation. Renugadevi et al. (2012) studied the *in vitro* cytotoxicity effect of AgNPs against Hep2 cell and Vero cell line at

different concentrations (20, 40, 60, 80, 100, 120 and 140 µg). They found that concentration required for 50% cell death ($IC_{50}$) for HEP2 and Vero cell line were 86 and 107 µg, respectively and stated that $IC_{50}$ value was found to be less for the Hep2 cell line than the Vero cell line. The effect of AgNPs on human epidermoid larynx carcinoma cell line exhibits a dose dependent toxicity for the cells tested (Satyavani et al., 2012) and the viability of Hep-2 cells decreased to 50% ($IC_{50}$) at the concentration of 500 µM. Toxicity of AgNPs on lung cancer cells (A549) and normal healthy peripheral lymphocytes (PLs) at 10 and 50 µg/ml was assessed using the MTT, ATP and lactate dehydrogenase assays (Gengan et al., 2013). They reported that A549 cells showed a 21% (10 µg/ml) and 73% (50 µg/ml) cell viability after 6 h exposure to AgNPs, whereas 117% (10 µg/ml) and 109% (50 µg/ml) cell viability in normal peripheral lymphocytes. Lactate dehydrogenase was only significantly altered at 50 µg/ml AgNPs treated cells.

## HIV-1 INHIBITION

It was noticed that AgNPs undergo size dependent interaction with HIV-1. NPs within the size range of 1 to 10 nm readily interact with the HIV-1 virus via preferential binding to gp 120 glycoprotein knobs. The specific interaction of the AgNPs inhibits the virus from binding to host cells (Elechiguerra et al., 2005). This provides evidence that AgNPs can prevent and control HIV infections.

## INSECTICIDAL AND LARVICIDAL ACTIVITIES

The larvicidal effect of *Mimosa pudica* aqueous leaf extract, silver nitrate solution and synthesized AgNPs against the larvaes of malaria vector (*Anopheles subpictus*) and filariasis vectors (*Culex quinquefasciatus* and *Rhipicephalus microplus*) was studied (Marimuthu et al., 2011). Parasite larvae were exposed to varying concentrations extracts for up to 24 h and found that the highest mortality rate was with AgNPs. The lethal concentrations of *A. subpictus, C. quinquefasciatus* and *R. microplus* larvae were ($LC_{50}$=13.90, 11.73, and 8.98 mg/L, r2=0.411, 0.286 and 0.479), respectively. The acaricidal and larvicidal activity of synthesized AgNPs utilizing aqueous leaf extract of *Musa paradisiaca* was tested against the larvae of *Haemaphysalis bispinosa* and larvae of hematophagous fly (*Hippobosca maculata*) and the fourth-instar larvae of malaria vector (*Anopheles stephensi*), Japanese encephalitis vector (*Culex tritaeniorhynchus*) (Jayaseelan et al., 2012). The green synthesized AgNPs of *M. paradisiaca* showed significant effect towards all the parasite vectors with the $LC_{50}$ and r2 values against *H. bispinosa*, (1.87 mg/L; 0.963), *H. maculata* (2.02 mg/L; 0.976), and larvae of *A. stephensi* (1.39 mg/L; 0.900), against *C. tritaeniorhynchus* (1.63 mg/L; 0.951), respectively.

The larvicidal activity of AgNPs synthesized using *Euphorbia hirta* leaf extract against malarial vector (*Anopheles stephensi*) was studied (Priyadarshini et al., 2012). AgNPs show the significant larval mortality against the first to fourth instar larvae and pupae and with the lethal concentrations ($LC_{50}$ = 10.14, 16.82, 21.51, 27.89 and 34.52 ppm, and $LC_{90}$ = 31.98, 50.38, 60.09, 69.94 and 79.76 ppm) respectively. Kamaraj et al. (2012) investigated feeding deterrent activity of AgNPs synthesized using *Manilkara zapota* leaf extract against *Musca domestica*. Adult flies were exposed to different concentrations of synthesized AgNPs for 1, 2 and 3 h and found that 100% of mortality within 3 h of duration at a concentration of 10 mg/L. The $LD_{50}$ and $LD_{90}$ values of AgNPs towords *M. domestica* were 3.64 and7.74 mg/ml, respectively. The larvicidal potential of AgNPs synthesized using aqueous leaf extract of *Nelumbo nucifera* against fourth instar larvae of *A. subpictus* and *C. quinquefasciatus* were investigated (Santhoshkumar et al., 2011). The maximum efficacy was found against the larvae of *A. subpictus* ($LC_{50}$= 0.69 ppm; LC90=2.15 ppm) followed by the larvae of *C. quinquefasciatus* (LC50= 1.10 ppm; $LC_{90}$= 3.59 ppm), respectively. AgNPs synthesized by using aqueous leaf extract of *Ocimum canum* were assessed for antiparasitic activity against the larvae of *Hyalomma anatolicum* and *Hyalomma marginatum*. The maximum efficacy was noticed against *H. anatolicum* followed by *H. marginatum* with $LC_{50}$ and $LC_{90}$ values of 0.78 and 1.00 and 1.51 and 1.68 mg/L, respectively (Jayaseelan and Rahuman, 2012).

Synthesized AgNPs from *Pedilanthus tithymaloides* were investigated for their efficacy against the dengue vector (*Aedes aegypti)* by exposing the larvae to varying concentrations of AgNPs for 24 h (Sundaravadivelan and Nalini, 2012). AgNPs showed 100% mortality from first to fourth instars and pupae of *A. aegypti* at 0.25%. Lethal concentrations ($LC_{50}$) values of AgNPs against the larval and pupal stages were found to be 0.029, 0.027, 0.047, 0.086 and 0.018%, respectively. The bioactivity of latex of *Pergularia daemia* as well as synthesized AgNPs against the larval instars of *Aedes aegypti* and *Anopheles stephensi* was determined. The $LC_{50}$ and $LC_{90}$ values of AgNPs-treated against first, second, third and fourth instars of *A. aegypti* ($LC_{50}$ =4.39, 5.12, 5.66, 6.18; $LC_{90}$ = 9.90, 11.13, 12.40, 12.95 ppm) and *A. stephensi* ($LC_{50}$ = 4.41, 5.35, 5.91, 6.47; $LC_{90}$ = 10.10, 12.04, 13.05, 14.08 ppm) were found many fold lower than crude latex treated (Patil et al., 2012). The pediculicidal and larvicidal activity of synthesized AgNPs and aqueous leaf extract of *Tinospora cordifolia* against human head louse (*Pediculus humanus*) and fourth instar larvae of malaria vector (*A. subpictus*) and filariasis vector (*C. quinquefasciatus*) was studied. The maximum mortality was found with AgNPs against louse than *A. subpictus* and *C. quinquefasciatus* with lethal concentration ($LC_{50}$=12.46, 6.43 and 6.96 mg/L; $r^2$=0.978, 0.773 and 0.828), respectively. 33% of mortality at 5 min, 67% at 15

min and 100% after 1 h were observed (Jayaseelan et al., 2011). Larvicidal activity of synthesized AgNPs utilizing aqueous extract from *Eclipta prostrata* was investigated against fourth instar larvae of filariasis vector (*C. quinquefasciatus)* and malaria vector (*A. subpictus*). The maximum efficacy of AgNPs was noticed towords *C. quinquefasciatus* ($LC_{50}$ = 4.56 mg/L; $LC_{90}$ = 13.14 mg/L) followed by *A. subpictus* ($LC_{50}$ = 5.14 mg/L; $LC_{90}$ = 25.68 mg/L) respectively (Rajakumar and Rahuman, 2011). The Larvicidal activity of *Murraya koenigii* ethanol leaf extract and AgNPs synthesized were studied by Suganya et al. (2013). The maximum mortality was found with synthesized AgNPs, in both *A. stephensi* ($LC_{50}$ values of 10.82, 14.67, 19.13, 24.35, and 32.09 ppm and $LC_{90}$ values of 32.38, 42.52, 53.65, 63.51, and 75.26 ppm) and *A. aegypti* ($LC_{50}$ values of 13.34, 17.19, 22.03, 27.57, and 34.84 ppm and $LC_{90}$ values of 36.98, 47.67, 55.95, 67.36, and 77.72 ppm), respectively.

## PESTICIDAL ACTIVITY

The efficacies of all the agents (aqueous leaves extracts of *Euphorbia prostrata*, silver nitrate ($AgNO_3$) solution (1 mM) and synthesized AgNPs were tested against the adult *Sitophilus oryzae* for 14 days to study their pesticidal activity. The $LD_{50}$ values of aqueous extract, $AgNO_3$ solution and synthesized AgNPs were found to be 213.32, 247.90, 44.69 mg/kg$^{-1}$; $LD_{90}$=1648.08, 2675.13, 168.28 mg/kg$^{-1}$, respectively (Zahir et al., 2012). E. prostrata synthesized AgNPs against the adult H. bispinosa showed LC50 at 2.30 ppm and LC90 value at 8.28 ppm where as against H. maculata they showed LC50 at 2.55 ppm and LC90 at 9.03 ppm respectively. Mortality of 100% was found in synthesized AgNPs at a concentration of 10 mg l$^{-1}$ (Zahir and Rahuman, 2012).

## CONCLUSION

Recent research works regarding the photo-biological approach for the synthesis of silver nanoparticles were summarized in the present review. Nature itself is a nano-factory; in nature, a number of plant species have potentiality to produce nanoparticles. In this review also, research work regarding the mechanisms involved in the phytosynthesis of silver nanoparticles is elucidated. Studies regarding the phytochemicals and factors that are responsible for the synthesis and stabilization of nanoparticles were briefly summarized. It is important to understand the complete mechanism involved in the biosynthesis (using plants) of silver nanoparticles for scaling up the process. Several biologists proposed numerous hypotheses regarding the mechanisms involved in plant mediated synthesis; it may be due to complex nature of plant materials. Therefore, further studies would be required to understand the particular mechanism involved in a specific plant species.

In the present decade interest in AgNPs applications has increased mainly because of the important antimicrobial activities of these nanoparticles, allowing their use in several industrial sectors. However, together with these applications, there is increasing concern related to the biological impacts of the use of silver nanoparticles on a large scale, and the possible risks to the environment and health. So, the investigation of potential inflammatory effects and diverse cellular impacts of silver nanoparticles is important. Another important issue related to nanoparticle toxicity is damage to the genetic material, because AgNPs are able to cross cell membranes and reach the cellular nucleus. Little is known about the genotoxicity of AgNPs and their effects on the DNA of organisms; up to the present literature, available biogenic silver nanoparticles are generally less cyto/genotoxic *in vivo* compared with chemically synthesized nanoparticles. However, further studies would be required to carefully analyze nanoparticles toxicity in order to decrease the possible discrepancies related to final conclusions.

## Conflict of Interests

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

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*Full Length Research Paper*

# Effects of *Moringa oleifera* Lam. aqueous leaf extracts on follicle stimulating hormone and serum cholesterol in Wistar rats

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**The study evaluated the effect of *Moringa oleifera* aqueous leaf extracts on follicle stimulating hormone and serum cholesterol in Wistar rats. Thirty six (36) mature Wistar rats (20 male and 16 female rats) were used. The male rats were grouped into four groups with five animals each, while the female animals were grouped into four made up of four animals per group, on the basis of their body weights. Graded doses (1, 5 and 10 g) aqueous extract were prepared from the *Mo* leaves as the test samples. In the first phase: The test extract was administered orally after acclimatization to individual groups: A-male and E-female rats, 1%; groups B-male and F-female rats, 5%; and groups C-male and F-female rats, 10%. These test groups also had rat chow and water *ad libitum.* The second phase of the experiment involved mating the male and female animals that had the same dose of *M. oleifera* extract. The results show that the mean body weights of the male rats increased significantly after treatment ($p<0.05$). The study also reveals that the administration of *M. oleifera* extract at different doses for the male and female rats differed significantly ($p<0.05$) with that of the control in raising the level of follicle stimulating hormone (FSH). A-1% increased by 38.52% while B-5% decreased (-21.20%); E-1% decreased (-12.96%) and F-5% (-25.64%). After mating, the % increase in FSH concentration was observed to be significantly ($p<0.05$) difference. Administration of Mo extract at different levels for the male and female rats differed significantly ($p<0.05$) in this study as compared with the control in lowering the total serum cholesterol of the rats. A-1% increased total cholesterol by 1.10%, while B-5% revealed a decrease of (-10.99%).**

**Key words:** *Moringa oleifera*, follicle stimulating hormone, serum cholesterol, Wistar rats.

## INTRODUCTION

The therapeutic use of *Moringa oleifera* parts in the Indian subcontinent dates back to antiquity. This plant is known as, a remedy to malnutrition and a vast range of ailments. *M. oleifera* is variably labelled as “Saviour of the Poor” (Mbikay, 2012). In many parts of Africa, it is widely consumed for self-medication by patients suffering from

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diabetes, anaemia, hypertension, or HIV/AIDS (Monera and Maponga, 2010; Otitoju et al., 2014; Beckman et al., 2000; Rotimi et al., 2011).

Besides culinary and other domestic uses, several biological properties ascribed to various parts of this plant have been reviewed in the past (Fahey, 2005). The leaves of *M. oleifera* have been reported to be a valuable source of both macro- and micronutrients, rich source of β-carotene, protein, vitamin C, calcium, and potassium and act as a good source of natural antioxidants; and thus enhance the shelf-life of fat-containing foods (Siddhuraju and Becker, 2003; Dillard and Bruce, 2000).

Follicle-stimulating hormone (FSH) is a glycoprotein gonadotropin secreted by the anterior pituitary in response to gonadotropin-releasing hormone (GnRH), which is released by the hypothalamus (Bowen, 2004). FSH is composed of alpha and beta subunits. The specific beta subunit confers the unique biological activity. FSH and luteinizing hormone LH bind to receptors in the testis and ovary and regulate gonadal function by promoting sex steroid production and gametogenesis (Grover et al., 2005). In women, follicle stimulating hormone stimulates the growth of ovarian follicles in the ovary before the release of an egg at ovulation, and promotes oestradiol production. In men, follicle stimulating hormone acts on the Sertoli cells of the testes to promote sperm production (spermatogenesis). Follicle stimulating hormone is one of the hormones essential to pubertal development and function of the gonads (ovaries and testes) both in women and men.

Cholesterol is a ubiquitous component of all animal tissues, where much of it is located in the membranes, although it is not evenly distributed (Maxfield and van Meer, 2010; Athenstaedt and Daum, 2006). In plants, it tends to be a minor component only of a complex mixture of structurally related phytosterols, although there are exceptions, but it is nevertheless important as a precursor of some plant hormones (John et al., 2007).

Within cells, cholesterol is the precursor molecule in several biochemical pathways (Smith, 1991). Cholesterol is an important precursor molecule for synthesis of vitamin D and the steroid hormones, including the adrenal gland hormones cortisol and aldosterone, as well as the sex hormones: progesterone, oestrogen and testosterone and their derivatives (Smith, 1991).

*M. oleifera* is reportedly used to alleviate menstrual pains; other reports implicated the same herb in causing abortion in rats hence its abonificient property. It has also been reported to be use in curing infertility; the actual effect of *M. oleifera* on the reproductive system and its hormones has not been fully investigated. Therefore, the objective of this study was to examine the effects of leaf extracts of *M. oleifera* on total cholesterol and follicle stimulating hormone.

## MATERIALS AND METHODS

Mature Wistar rats (20 males and 16 females) were used for this study. Choice of different numbers of animals was to reduce competition among the males and for effective reproductive experiment. The animals were procured from the Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Nigeria.

The *M. oleifera* leaves were gotten from the demonstration farm of Crop Science Department, University of Nigeria, Nsukka, pulverized and aqueous extract produced from them. The samples of *M. oleifera* leaves were shade-dried and subsequently ground to powder using household blender. Aqueous extract was prepared by adding 1 g of *M. oleifera* leaf to 99 ml of $H_20$; 5 g of *Mo* leaf to 95 ml of $H_20$; 10 g of *Mo* leaf to 90 ml of $H_2O$ and each solution labelled.

### Chemical and bio-chemicals

All the chemicals that were used in the research were of analytical grade. Total cholesterol assay, Randox Monza CH 200 kit was used; manufactured by Randox Laboratories Limited United Kingdom. For follicle stimulating hormone assay, Accu-Bind ELISA Microwells kit was used; manufactured by Monobind Incorporated United States of America. Chloroform, formalin was also used.

### Animal treatment

Twenty (20) mature males and 16 female Wistar rats aged 10 weeks were weighed and divided into groups according to their weights; 3 test and control groups containing 5 rats each for males while the females had 4 rats each. The rats were kept in separate cages and labelled according to groups. They were acclimatized for a period of one week in the metabolic cages in the Department of Home Science, Nutrition and Dietetics, University of Nigeria Nsukka. They were fed with Vita feed Finisher (rat chow) and water *ad libitum*. The study lasted for a total duration of 51 days comprising of two phases.

#### *Phase 1*

After acclimatization, the rats were fed with rat chow and water *ad libitum*, and treated with test leaf extract A-1%, B-5% and C-10% for 14 days (these concentrations were below the $LD_{50}$ of *M. oleifera;*15.9 g/kg body weight). The extract was administered using oral gavage except for the control group who had only rat chow and water *ad libitum*. At the end of the first phase, the rats were anesthetized and blood samples collected and analysed to determine the level of follicle stimulating hormone and total cholesterol concentration.

#### *Phase 2*

The animals were regrouped for mating, males introduced to females who were administered the same concentration of *M. oleifera* and monitored for 30 days. At the end of the duration, the rats were anaesticized with chloroform and blood samples collected and analysed to determine the follicle stimulating hormone and total cholesterol concentrations.

### Sample collection

Overnight before the days of sample collection, the animals were fasted of solid food. Blood (5 ml) was collected from the ocular *median-cantus* vein of the rats with the aid of capillary tubes and transferred into sample bottles containing no EDTA. The samples were allowed to clot and centrifuged under cold condition at 4000 rpm in a table top centrifuge. The serum layers were collected for the hormone assay.

**Table 1.** Mean feed intake in grams of male and female Wistar rats that were administered graded doses of *M. oleifera* aqueous leaf extracts.

| Male group | Feed intake (g) | Female group | Feed intake (g) |
|---|---|---|---|
| A-1% | $70.33^{b}$±14.03 | E-1% | $61.07^{b}$±9.47 |
| B-5% | $62.80^{b}$±17.09 | F-5% | $52.33^{b}$±14.27 |
| C-10% | $51.00^{b}$±17.20 | G-10% | $54.20^{b}$±9.64 |
| D-control | $151.60^{b}$±20.21 | H-control | $110.33^{b}$±34.09 |

Mean scores with the same superscripts are statistically different at $p < 0.05$. Values are mean±SD of five variants for male and four variants for female. A, Male group, 1% of *M. oleifera* aqueous leaf extract was administered; B, male group, 5% of *M. oleifera* aqueous leaf extract was administered; C, male group, 10% of *M. oleifera* aqueous leaf extract was administered; D, male control group, no *M. oleifera* aqueous leaf extract was administered; E, female group, 1% of *M. oleifera* aqueous leaf extract was administered; F, female group, 5% of *M. oleifera* aqueous leaf extract was administered ;G, female group, 10% of *M. oleifera* aqueous leaf extract was administered; H, female control group, no *M. oleifera* aqueous leaf extract was administered.

**Table 2.** Body weight of Wistar rats fed graded doses of *M. Oleifera* aqueous leaf extract and % Mean Increase at end of the third Week.

| Male group | Mean | Increase (%) | Female group | Mean | Increase (%) |
|---|---|---|---|---|---|
| A-1% | 269.16±22.26 | 4.40 | E-1% | 157.73±39.54 | 8.47 |
| B-5% | 223.72±71.94 | 46.40 | F-5% | 142.02±7.23 | 14.84 |
| C-10% | 233.38±40.84 | 30.38 | G-10% | 147.01±2.59 | 15.33 |
| D-control | 221.34±29.85 | 1.07 | H-control | 156.59±16.13 | 1.38 |

Mean scores with the same superscripts are statistically different at $p < 0.05$. Values are mean±SD of five variants for male and four variants for female. A, Male group, 1% of *M. oleifera* aqueous leaf extract was administered; B, male group, 5% of *M. oleifera* aqueous leaf extract was administered; C, male group, 10% of *M. oleifera* aqueous leaf extract was administered; D, male control group, no *M. oleifera* aqueous leaf extract was administered; E, female group, 1% of *M. oleifera* aqueous leaf extract was administered; F, female group, 5% of *M. oleifera* aqueous leaf extract was administered; G, Female group, 10% of *M. oleifera* aqueous leaf extract was administered; H, female control group, no *M. oleifera* aqueous leaf extract was administered.

#### Hormone assay

The concentrations of follicle stimulating hormone and total cholesterol were determined in the serum. The concentration of follicle stimulating hormone was carried out using Accu-bind Enzyme Linked Immunosorbent Assay kits from Monobind Incorporated, Lake Forest, USA. Total cholesterol concentration was spectrophotometrically determined using Randox cholesterol kits.

#### Assay principle

The cholesterol was determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine was formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidise.

#### Data analysis

The mean, standard deviation and analysis of variance (ANOVA) test were used as the statistical tools for analysing the results between the treated groups and control groups.

## RESULTS

Table 1 shows the mean feed intake by the different male and female groups of Wistar rats that were administered graded doses of *M. oleifera* aqueous leaf extract. Group A treated male rats with 1% *M. Oleifera* leaf extract had the highest mean feed intake but not compared to the control hence was significantly ($p<0.05$) different.

For female Wistar rats that were administered graded doses of *M. oleifera* aqueous leaf extract; the result showed that group E administered 1% *M. oleifera* aqueous leaf extract consumed 61.07 g of the feed, a value highest among the treated groups but not compared to the control. There was significant difference ($p<0.05$) between the means of the various female groups.

Table 2 shows the mean body weight (g) of Wistar rats that were fed graded doses of *M. oleifera* aqueous leaf extract. Male rats in group B that were administered 5% of *M. oleifera* aqueous leaf extract showed more weight gain of 46.40% than C fed 10% *Mo* (30.38%) at the end of the third week. Females in group F (5%) were observed to have weight gain of 14.84% than G 10% with 15.33%.

Table 3 shows the mean concentration in mIU/ml of follicle stimulating hormone (FSH) of male and female Wistar rats that were administered graded doses of *M. oleifera* aqueous leaf extracts. Male rats in group A that were administered 1% of *M. oleifera* aqueous leaf extract

**Table 3.** Concentration of follicle stimulating hormone of male and female Wistar rats that were administered graded doses of *M. oleifera* aqueous leaf extracts.

| Male group | FSH conc. (mIU/ml) | Increase (%) | Female group | FSH conc. (mIU/ml) | Increase (%) |
|---|---|---|---|---|---|
| A-1% | $3.92^{a}$±2.28 | 38.52 | E-1% | $2.38^{b}$±0.30 | -12.96 |
| B-5% | $2.23^{b}$±0.12 | -21.20 | F-5% | $2.03^{c}$±0.05 | -25.64 |
| C-10% | $3.50^{a}$±0.00 | 23.67 | G-10% | $2.55^{b}$±0.52 | -6.59 |
| D-Control | $2.83^{b}$±0.15 | | H-control | $2.73^{a}$±0.25 | |

Mean scores with the same superscripts are statistically different at $p < 0.05$. Values are mean±SD of five variants for male and four variants for female. A, Male group, 1% of *M. oleifera* aqueous leaf extract was administered; B, male group, 5% of *M. oleifera* aqueous leaf extract was administered; C, male group, 10% of *M. oleifera* aqueous leaf extract was administered; D, male control group, no *M. oleifera* aqueous leaf extract was administered; E, female group, 1% of *M. oleifera* aqueous leaf extract was administered; F, female group, 5% of *M. oleifera* aqueous leaf extract was administered; G, female group, 10% of *M. oleifera* aqueous leaf extract was administered; H, female control group, no *M. oleifera* aqueous leaf extract was administered.

**Table 4.** Concentration (Mmol/l) of total cholesterol (TC) of male and female Wistar rats that were administered graded doses of *M. oleifera* aqueous leaf extract.

| Male group | TC conc. (Mmol/L) | Increase (%) | Female group | TC conc. (Mmol/L) | Increase (%) |
|---|---|---|---|---|---|
| A-1% | $2.76^{a}$±0.43 | 1.10 | E-1% | $2.65^{c}$±0.19 | -4.33 |
| B-5% | $2.43^{b}$±0.50 | -10.99 | F-5% | $2.35^{b}$±0.24 | -15.16 |
| C-10% | $2.55^{b}$±0.07 | -6.59 | G-10% | $2.10^{c}$±0.08 | -24.19 |
| D-control | $2.73^{a}$±0.40 | | H-control | $2.77^{a}$±0.45 | |

Mean scores with the same superscripts are statistically different at $p < 0.05$. Values are mean±SD of five variants for male and four variants for female. A, Male group, 1% of *M. oleifera* aqueous leaf extract was administered; B, male group, 5% of *M. oleifera* aqueous leaf extract was administered; C, male group, 10% of *M. oleifera* aqueous leaf extract was administered; D, male control group, no *M. oleifera* aqueous leaf extract was administered; E, female group, 1% of *M. oleifera* aqueous leaf extract was administered; F, female group, 5% of *M. oleifera* aqueous leaf extract was administered; G, female group, 10% of *M. oleifera* aqueous leaf extract was administered; H, female control group, no *M. oleifera* aqueous leaf extract was administered.

had the highest concentration of FSH (3.92 mIU/ml) with a percentage increment of 38.52%, followed by the rats in group C that were administered 10% of *M. oleifera* (3.50 mIU/ml) (23.67%) increase. The rats in group B which were administered 5% *M. oleifera* leaf extract had the lowest FSH concentration (2.23 mIU/ml) with a decrease of 21.20%. There was significant statistical difference ($p<0.05$) between the mean FSH concentration of the groups compared with control group.

Table 4 shows the mean concentration of total cholesterol of Wistar rats that were administered graded doses of *M. oleifera* aqueous leaf extract. Group A which was administered 1% of *M. oleifera* aqueous leaf extract, had the highest mean concentration, of total cholesterol (2.76 Mmol/L), with a percentage increase of 1.10% while group B which was given 5% of *M. oleifera* aqueous leaf extract had 2.43 Mmol/L with a decrease mean percentage of -10.99%. There was statistically significant difference ($p<0.05$) in the mean concentration of total cholesterol between the group.

## DISCUSSION

This study reveals that during administration of the aqueous *M. oleifera* leaf extract, as shown in Table 1, aqueous *M. oleifera* leaf extract had significant ($p<0.05$) effect on the feed intake of the male Wistar rats. This shows that aqueous *M. oleifera* leaf extract altered the appetite and subsequently the weight of the rats. This confirms the reports by Chandra (2012) and Oyewo (2013) who stated that one of the reasons behind hunger is decreased amounts of vitamins, minerals and other needed nutritional elements. Because *M. oleifera* is so nutritionally dense, it provides many of these without a large amount of calories. This can keep the hunger urges from striking when they are unwanted. Based on the fact that the leaves themselves provide 42% of one's recommended daily protein, you will feel full and get the vitamins and minerals you need.

In addition to catering for basic nutritional needs, *M. oleifera* contains a unique blend of antioxidants and complex proteins that work together to provide a host of health benefits, including mental clarity, improved feelings of emotional wellbeing, increased energy and stamina. Many people who have weight problems found out that the enhanced emotional feelings go a long way in keeping away cravings for certain sugary foods (Chandra, 2012).

The use of *M. oleifera* as a potential herb for the treatment of infertility among its consumers is on the high

increase with antecedents of complaints relating to various observations such as irregular menstruation, loss of menstruation, heavy menstrual flow, and decrease sexual urge especially in men. The decrease in the concentrations of FSH gives a lot of information with respect to its biological functions. Since FSH and luteinising hormone LH bind to receptors in the testis and ovary and regulate gonadal function by promoting sex steroid production and gametogenesis (Grover et al., 2005), any decrease may in turn decrease its function relating to gametogenesis. Biologically in men, follicle stimulating hormone acts on the Sertoli cells of the testes to promote sperm production (spermatogenesis). Follicle stimulating hormone is also one of the hormones essential to pubertal development and function of the gonads (ovaries and testes) both in women and men (Grover et al., 2005).

In women, follicle stimulating hormone stimulates the growth of ovarian follicles in the ovary before the release of an egg at ovulation, and promotes oestradiol production (Grover et al., 2005). The rise in follicle stimulating hormone stimulates the growth of the follicle in the ovary. With this growth, the cells of the follicles produce increasing amount of oestradiol and inhibin (en.wikipedia.org/Wiki/Hypothalamic-pituitary-gonada-axis, cited 2014). The findings of the study also reveals that the administration of *M. oleifera* extract at different percentages for the male and female Wistar rats differed significantly ($P<0.05$) with that of the control in raising the follicle stimulating hormone (FSH) even after they were regrouped for mating. However, rats administered with the high and medium doses of *M. oleifera* leaf extract are reproductively superior to those that were given low doses and no dose. This is in contrast with a similar work done by Cajuday and Pocsido (2010) where they found out that the effect of *M. oleifera* had clearly manifestations enhancing male reproduction in all the treated groups compared with the control. Sudwan et al. (2007) found that the ethanolic extract of another plant, *Boesenbergia rotunda,* does not affect sexual behavior or serum androgen levels, but enhances seminiferous tubule, testis and seminal vesicle in the treated male rats. Similarly, Watcho et al. (2001) found that the rats treated with *Mondia whitei* for eight days induced an increase in the testicular weight, testicular testosterone level and sperm density without affecting the accessory gland weights whereas, Salman et al. (2013) and Gonzales et al., (2001) observed that rats treated with honey had significantly higher sperm counts compared to those in control group but there were no significant differences for sperm morphology, seminiferous tubule diameter, weights of reproductive organs and in the levels of reproductive hormones.

The loss in weight with increase in *M. oleifera* leaf extract implicates the product as agent of anti-obesity as reported by other studies which is a consequence hypocholesterolemia (Ghasi et al. 2000).

## Conclusion

In conclusion, prolong oral and possible high dosages administration of aqueous leaf extract of *M. oleifera* may adversely affect the production process of follicle stimulating hormone. The observed cholesterol-reducing action of the crude leaf extract of *M. oleifera* indicates that this leafy vegetable possesses some potential medicinal value and could validate and explain its ethno-medicinal use on the obese and heart disease patients, but this observed positive effect may affect reproductive capacity if consumed over a long period of time.

## Conflict of Interests

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

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# Effect of different hydrolysis methods on starch degradation

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**Hydrolysis of cassava starch was carried out using different processing routes namely: malt extract; acid; combinations of α-amylase and amyloglucosidase; combinations of acid, α-amylase and amyloglucosidase and combinations of malt extract, α-amylase and amyloglucosidase. The results of hydrolysis of all the five routes shows a wide degree of variance in their susceptibility to acid/enzyme hydrolysis in starch conversion of malt extract; acid; combinations of α-amylase and amyloglucosidase; combinations of acid, α-amylase and amyloglucosidase; combinations of malt extract, α-amylase and amyloglucosidase with dextrose equivalent (DE) of 24.29; 33.33; 73.43; 61.29; 76,74 DE, respectively, except the combinations of α-amylase and amyloglucosidase and combinations of malt extract, α-amylase and amyloglucosidase that shows very closed range. The best result was obtained with hydrolysis of the combinations of malt extract, α-amylase and amyloglucosidase which was observed to be more efficient than using any of the other routes in the present work.**

**Key words:** Degradation, routes, α-amylase, glucoamylase, hydrolysis, dextrose equivalent (DE).

## INTRODUCTION

Starch is laid down in all higher plants in the form of bi-refringent, and semi-crystalline granules. These granules are primarily composed of two glucose polymers, essentially linear amylose and highly branched amylo-pectin. The granule crystallinity is associated with the amylopectin component (Montgomery and Senti, 1958; Meyer, 1942). The currently accepted amylopectin structure involves short amylopectin chains forming double helices and associating into clusters (Robin et al., 1974a, 1975b). These clusters pack together to produce a structure of alternating crystalline and amorphous lamellar composition (Figure 1). Regions of amylopectin double helix formation fall within the crystalline lamellae, whilst the amylopectin branch points lie in the amorphous lamellae. In the model detailed in Figure 1, the lamellae are oriented perpendicular to the helix axis. It has also been suggested that the lamellae may be inclined with respect to the helix axis (Oostergetel and Van Bruggen, 1987; Van Breemen et al., 1994). This possibility is not considered in this paper. For the amylopectin within the crystalline lamellae, three different types of crystalline structure, labelled A, B and C, are identified by wide angle X-ray scattering. A connection has been esta-blished between the crystalline structure and the length

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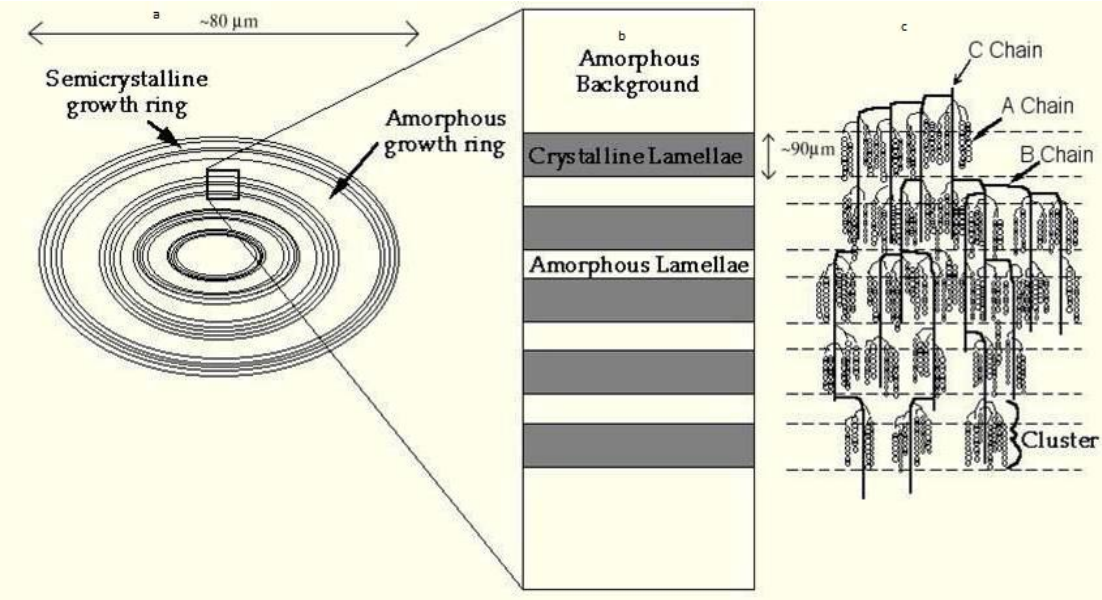


**Figure 1.** Schematic diagram of the structure of a starch granule. **(a)** Single granule comprising concentric rings, each containing stacks of amorphous and crystalline lamellae. **(b)** Amorphous and crystalline lamellae**. (c)** Chains of amylopectin arranged in a cluster structure (Jenkins and Donald, 1995).

of the amylopectin chains forming the clusters (labelled as A chains) (Hizukuri et al., 1983; Hizukuri, 1985).

Short A-chains are associated with A-type crystallinity, longer A-chains display B-type crystallinity, whilst intermediate-length A-chains show C-type crystallinity. The combined size of the crystalline plus amorphous lamellae is 9 nm (Jenkins et al., 1993) within the granule stacks of the amorphous and crystalline lamellae form radial rings (growth rings), around 120-400 nm thick (Yamaguchi et al., 1979), bounded by amorphous rings of a similar size. The precise structural role played by amylose is unclear. It is likely that a large portion is found within the amorphous growth ring, with only small amounts associated with the semi-crystalline growth ring 1. It has been suggested that some amylase co-crystallizes with amylopectin within the crystalline lamella (Blanshard, 1987; Kasemsuwan and Jane, 1994). Amylose may also form inclusion complexes with any lipids present internally within the starch granule.

Starch species exist with varying amylose and amylopectin contents. Naturally occurring starches typically have a range between 17% amylose (83% amylopectin) for tapioca and 28% amylose (72% amylopectin) for maize and wheat starch (Jenkins and Donald, 1995). Amylases are starch degrading enzymes. They are widely distributed in microbial, plant and animal kingdoms. They degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes. Initially the term amylase was used originally to designate enzymes capable of hydrolyzing α-1,4 -glucosidic bonds of amylose, amylopectin, glycogen and their degradation products (Bernfeld, 1955; Fisher and Stein, 1960; Myrback and Neumuller, 1950). They act by hydrolyzing bonds between adjacent glucose units, yielding products characteristic of the particular enzyme involved. In recent years a number of new enzymes associated with degradation of starch and related polysaccharides structures have been detected and studied (Buonocore et al., 1976; Griffin and Fogarty, 1973).

The objective of this work was to carry out hydrolysis on cassava starch using different routes with a view to identifying optimal processing method. Considerable work on cassava starch hydrolysis has been done (Aderibigbe, 2005; Solomon et al., 2006; Aderibigbe, 2011; Anozie and Aderibigbe, 2011), suggested various hydrolytic procedures for starch hydrolysis, designed and fabricated a pilot plant for future starch conversion to fermentable sugars. The malt extract has essential hydrolytic actions of endogeneous enzymes, mainly amylases and proteases, which convert insoluble carbohydrates and proteins into fermentable sugars and amino acids (Aderibigbe et al., 2012a). α-Amylase is used to pre-thin the gelatinized starch. Next, glucoamylase (amyloglucosidase) hydrolyzes α-1,4 links releasing glucose molecules from the non-reducing end of the chain. The α-1,6 branch links are also hydrolyzed but very much less rapidly.

## MATERIALS AND METHODS

### Cassava flour preparation

Fresh cassava harvested from a farm in Ede, Osun State, Nigeria

were peeled and washed with tap water, chipped into small sizes and dried at 60°C for 48 h in a cabinet dryer. The dried chips were dry-milled and screened to produce the flour. The flour was hydrolysed using a pilot plant designed and fabricated after the previous laboratory experiment (Aderibigbe, 2005).

**Malted sorghum preparation**

Sorghum bicolor invers (L. meench) also obtained from Timi market in Ede, Osun State of Nigeria and malted (Ilori et al., 1990). The sorghum grains were cleaned, then steeped in 500 ppm formaldehyde solution for 18 h with replacement of the steep liquor at 6 h intervals (Solomon et al., 1994). The steeped grains were germinated in a germination chamber at 28 ± 2°C, prior to germination; the chamber was disinfected with 1500 ppm formaldehyde solution. During germination the kernels were turned and wetted twice daily with steep liquor. The germination was carried out for 108 h that is, 4½ days after which it was terminated by kilning at 48 ± 20°C and milled to pass through 600 µm screen.

**Burtonized water preparation**

One hundred milliliters of salt slurry ($CaSO_4$, 235 g, $CaCl_2.H_20$, 75.5 g, $CaCL_2.7H_20$, 126 g, NaCl, 17 g, will be dissolved in 1 L of deionized water) will be added to 6.8 L of tap water to obtain the burtonized water (mineral water) (Briggs et al., 1986).

**Cassava flour hydrolysis with malt extract**

Samples of the sorghum malt of 17.5 kg weight were mixed with burtonized water to make 15 L, inside a hydrolyzer at different instances and heated to 50°C and held at that temperature, with constant stirring at 200 rpm, for 30 min. The residue sparged thrice with 5 L of burtonized water each time to extract the maximum amount of the malt enzymes. The enzyme liquor was kept at 50°C in a water bath. The residue was mixed with a seventeen and a half-kilograms (35% slurry) sample of cassava flour made to 20 L with burtonized water. The resulting mixtures were maintained at 50°C for 10 min. Thereafter, it was heated to 98°C, held at this temperature for another 10 min to gelatinize the starch, and then cooled to 64°C. The gelatinized starch slurry was then added to the extracted enzyme and the final mixture was kept at 64°C (the optimum temperature for β-amylase activity for a period of 1 h after which the temperature was raised to and maintained at 72°C (optimum temperature for α-amylase) activity for a further 1 h period with the pH varied from 4.5; 5.0, 5.5 to 6.0 to determine the optimum product level. Samples were withdrawn regularly and filtered after stopping the enzymatic activities by boiling for 15 min and analyzed for reducing sugar using Dinitrosalicylic method as described by Miller (1959).

The procedures described above for the case of malt to cassava flour ratio 1:1 were carried out in triplicates and only the average values were reported. The hydrolysis was similarly carried out with malt to cassava flour ratio 2:3.and only the average was reported.

**Acid hydrolysis of cassava flour**

A 17.5 kg cassava flour hydrolysis was dispatched into the hydrolyzer and conducted at different acid (HCL) concentrations of 0.05, 0.1, 0.2, 0.3 and 0.4 M and at temperature and agitation speed of 80°C and 250 rpm, respectively for a period of 180 min. Samples were drawn at regular time intervals and analyzed for reducing sugar using Dinitrosalicylic method as described by Miller (1959). The hydrolysis was carried out in triplicates and only the average values are reported.

**Cassava flour hydrolysis with α-amylase and glucoamylase**

A 17.5 kg (35 % slurry) cassava flour was dispatched into the hydrolyzer and cooked at 98°C for about 10 min so as to gelatinize the starch and a quantity of termamyl heat stable α-amylase from *Bacilus licheniformis* (equivalent to 0.5 mL/kg starch) was added and allowed to remain at this condition for 5 mn to thin out. It was cooled down to 72°C for another 25 min. The mixture was further cooled down to 60°C when a quantity of amyloglucosidase (equivalent to 0.5 mL/kg starch) was also added and allowed to remain at this condition for 2 h while the pH varied from 4.5; 5.0, 5.5 to 6.0 to determine the optimum product level. Samples were withdrawn regularly and filtered after stopping the enzymatic activities by boiling for 15 min and analyzed for reducing sugar using Dinitrosalicylic method as described by Miller (1959). These procedures were carried out in triplicate and only the average values are reported.

**Cassava flour hydrolysis with acid, α-amylase and glucoamylase**

A 17.5 kg cassava flour hydrolysis was dispatched into the hydrolyzer and the analysis was conducted at acid (HCL) concentrations of 0.4 M and as in “malted sorghum preparation” section followed by procedure of “Burtonized water preparation” section of this work. Samples were withdrawn regularly and filtered after stopping the enzymatic activities by boiling for 15 min and analyzed for reducing sugar using Dinitrosalicylic method as described by Miller (1959). The procedures were carried out in triplicate and only the average values are reported.

**Cassava flour hydrolysis with malt extract, α-amylase and glucoamylase**

A 17.5 kg (35% slurry) cassava flour was dispatched into the hydrolyzer and cooked at 98°C for about 10 min so as to gelatinize the starch and hydrolyze it with malt extract, α-amylase and glucoamylase as carried out in “cassava flour preparation” section and “Burtonized water preparation” section of this work. Similarly pH was varied from 4.5; 5.0, 5.5 to 6.0 to determine the optimum product level. Samples were withdrawn regularly and filtered after stopping the enzymatic activities by boiling for 15 min and analyzed for reducing sugar using Dinitrosalicylic method as described by Miller (1959). These procedures were carried out in triplicate and only the average values are reported.

## RESULTS AND DISCUSSION

The variation of reducing sugar concentration with time and pH using combinations of malt to cassava starch ratio 1:1 in 35% slurries is presented in Figure 2. It was observed that the reducing sugar concentration increased from pH 4.5 to 5.5 and dropped as pH increased to 6.0 but levelled off after 100 min. The maximum reducing sugar was about 85 g/L at pH 5.5. Figure 3 presents variation of reducing sugar concentration with time and malt to cassava starch ratios 1:1; 2:3 at pH 5.5 in 35% slurries respectively. It was observed that combinations with ratio 1:1 gave a better conversion than ratio 2:3 which was just about 50 dextrose equivalent (DE) compared with the yield of ratio 1:1. This could probably be possible because it contained enough malt extract to

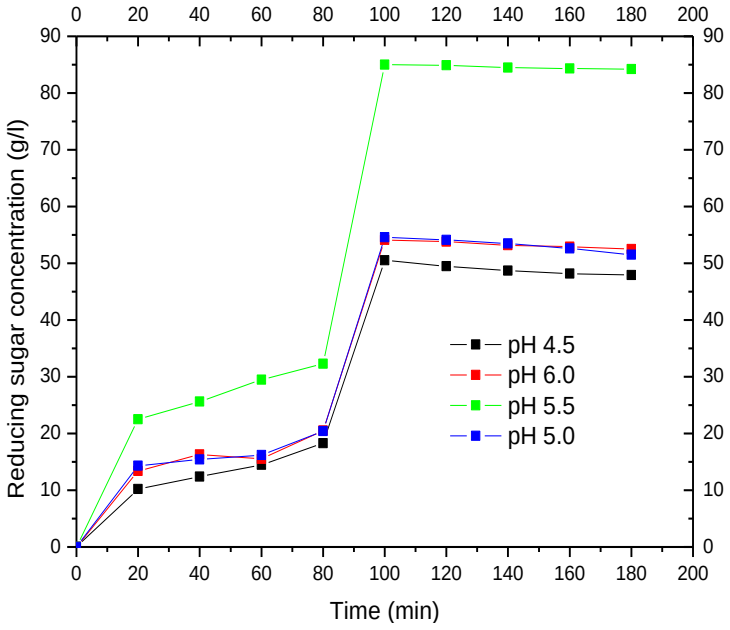


**Figure 2.** Variation of reducing sugar concentration with time in malt extract hydrolysis with cassava starch basis of 350 g/L.

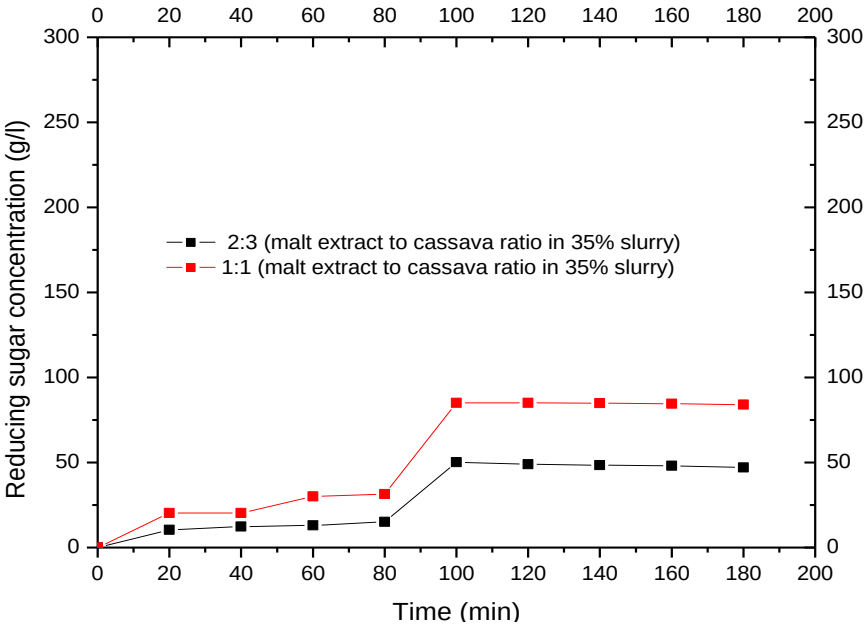


**Figure 3.** Variation of reducing sugar concentration with time in malt extract hydrolysis with different ratio of combination of malt to cassava at the optimum pH with cassava starch basis of 350 g/L.

hydrolyse the starch than 2:3 ratio combinations. The reducing sugar concentration increased with time over a period of study for the two cases but levelled off after 100 min. Figure 4 shows variation of reducing sugar concentration with acid concentration at 80°C. It was observed that the reducing sugar concentration increased with acid concentration from 0.05 to 0.4 M HCL but the curves levelled off after 100 min of hydrolysis. The DE at 0.4 M HCL was 51 and at 0.05 M HCL it was 19. In Figure 5 there were tremendous improvements in the

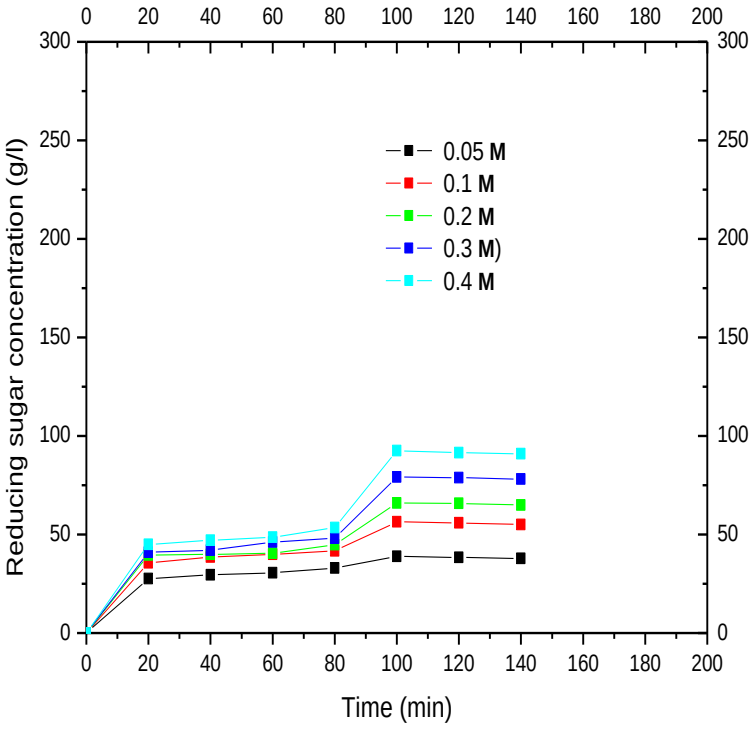


**Figure 4.** Variation of reducing sugar concentration with time in acid (HCl) hydrolysis with cassava starch basis of 350 g/L.

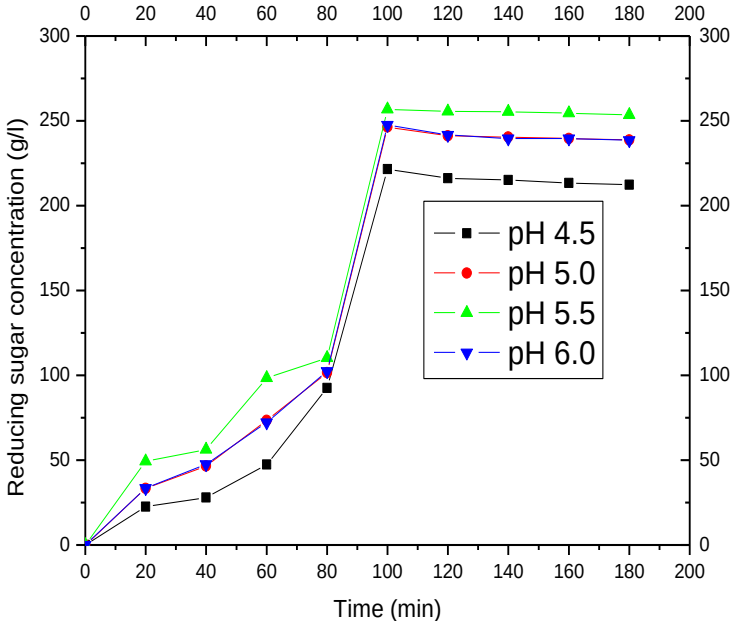


**Figure 5.** Variation of reducing sugar concentration with time in α-amylase and amyloglucosidase hydrolysis with cassava starch basis of 350 g/L.

starch conversion to a DE of 73.43 with the combinations of amylase and amyloglucosidase in the hydrolysis at pH 5.5. When acid was added to amylase and amyloglucosidase, the conversion drop to 61.29 DE which is significant as shown in Figure 6. Figure 7 further enhanced the starch conversion with the addition of malt extract to both amylase and amyloglucosidase to give overall best conversion of 76.74 DE. However, Figures 2

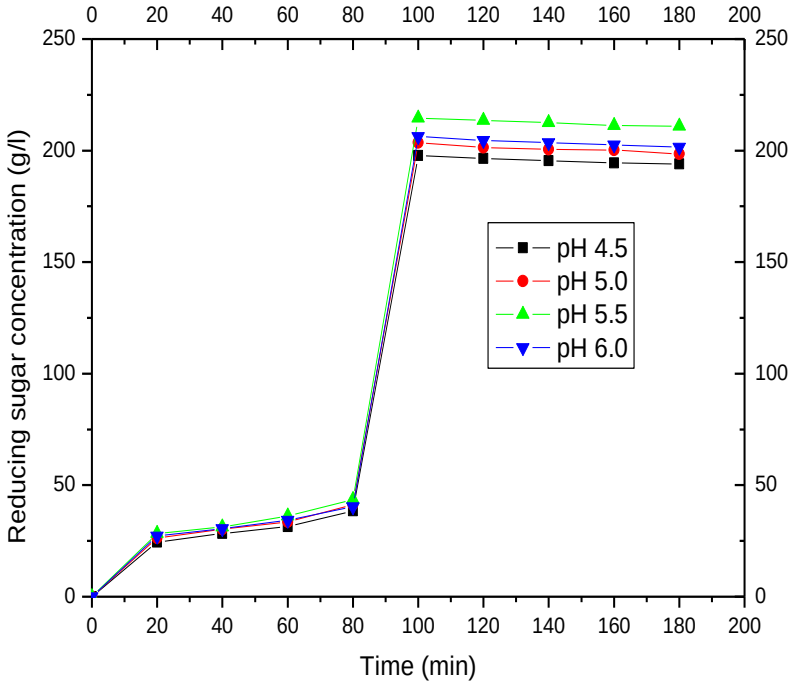


**Figure 6.** Variation of reducing sugar concentration with time in acid, α-amylase and amyloglucosidase hydrolysis with cassava starch basis of 350 g/L.

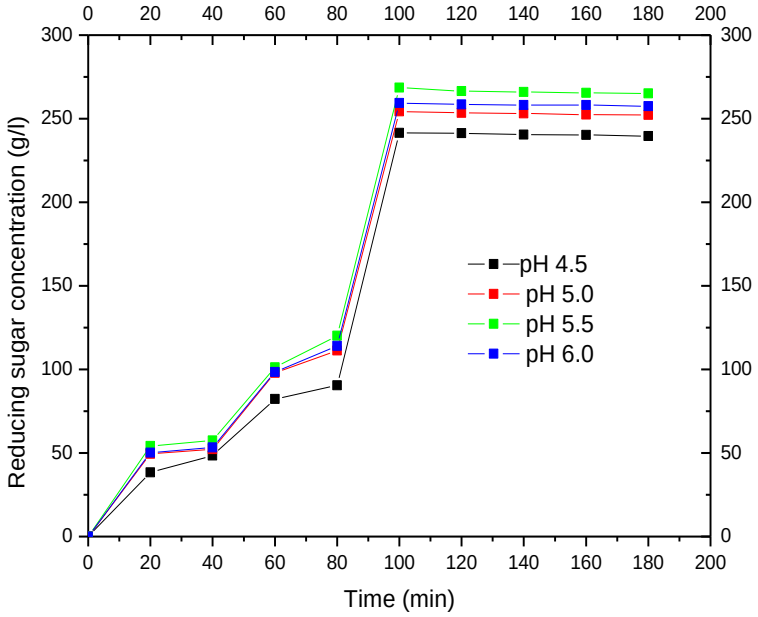


**Figure 7.** Variation of reducing sugar concentration with time in malt extract, α-amylase and amyloglucosidase hydrolysis with cassava starch basis of 350 g/L.

to 7 compared reducing sugar concentration with time in malt extract, acid; combinations of α-amylase and amyloglucosidase; combinations of acid, α-amylase and amyloglucosidase; combinations of malt extract, α-amylase and amyloglucosidase media hydrolysis, respectively. It was observed that malt extract; acid; combinations of amylase and amyloglucosidase; combinations of acid, α-amylase and amyloglucosidase; and combinations of malt extract, α-amylase and amyloglucosidase media gave about 24.29; 33.33; 73.43;

**Table 1.** Comparison of the DE at the Optimum Conditions in all the methods of hydrolysis

| Medium | DE |
|---|---|
| Malt extract | 24.29 |
| Acid | 33.33 |
| amylase + amyloglucosidase | 73.43 |
| Acid +amylase + amyloglucosidase | 61.29 |
| Malt extract + amylase + amyloglucosidase | 76.74 |

DE, Dextrose equivalent.

61.29 and 76.74 DE, respectively. Combinations of amylase and amyloglucosidase and combinations of malt extract, α-amylase and amyloglucosidase media gave best yields with 73. 34 and 76.74 DE, respectively. It was also observed that malt extract enhanced production of reducing sugar concentration and with a purer product compared with acid conversion.

Aderibigbe (2005) and Solomon et al. (2006) reported 21.67% conversion in 0.5 M dilute acid after 10 h, this is less by 11.66% to what is obtained in this work at the acid concentration of 0.4 M and at 80°C and 100 min. Azhar and Hamdy (1981) revealed from their work on acid hydrolysis of sweet potato that a maximum DE of 83 was reached after 18 min of hydrolysis using 0.1 M, HCI heated at 154°C. However, when the acid level was increased to 1.0 M, a rapid decrease in DE of 60 was noted within 6 min at 154°C followed by a marked decline to 42.5 DE after 12 min. Miller and Cantor (1952) reported that D-glucose formed during starch hydrolysis by acid was hydrated to 5-hydroxy-methyl furfural as the main product and lesser amount of hydroxyl-methyl furan. The acid conversion is higher and faster than malt extract conversion because the acid reaction went directly into the inner part of the starch granules while malt extract acted slowly at peripheral of the starch granules given purer products. The combinations of malt extract, amylase and amyloglucosidase medium gave the best overall yield compared with other processing routes adopted in this work.

Nebesyn (1990) reported a DE of 36.60 after 4 h of hydrolysis using 8 mg/mL of maltogenase. Ayernor et al. (2002) reported DE of 53.81 and 54.04 in combination of 8% w/v of rice malt extract with 300-unit/mL AMG and 10% w/v of rice malt extract with 200-unit/mL AMG after 2 h of hydrolysis. Nebesyn (1990) reported a DE of 22.60 after 4 h of hydrolysis with fungamyl α-amylase and a DE of 33.60 after 4 h of hydrolysis using fungamyl α-amylase with amyloglucosidase. There are a number of enzymes involved in the complete hydrolysis of starch to yield very high levels of sugars and each of these enzymes contribute only to some extent to the final yield and the type of sugars produced.

Table 1 compares the DE obtained in all the processing routes. The success of the combinations of malt extract with α-amylase and amyloglucosidase over other methods could be due to enzyme type, α–amylase and β-amylase present in malt extract which was able to hydrolyse α -1, 4 bonds and bypassed α -1, 6 linkages (endo-acting amylases) and hydrolyse α -1, 4 bonds and cannot bypassed α -1, 6 linkages (exo-acting amylases), respectively in amylopectin and related polysaccharides of the starch and amyloglucosidase activity which is also exo-acting enzyme and β-amylase, which rapidly break starch down starting from non-reducing end to release glucose and increase the yield of sugars. These enzymes are also weakly hydrolytic towards α -1, linkages, a fact of commercial importance since this activity permits the production of high glucose syrup (Tucker and Woods, 1991). Greater activity towards these linkages can be achieved by addition of more enzymes. However, this can result in the unwanted side reaction termed “reversion”, in which the glucose molecules produced repolymerise to form isomaltose, and hence the final yield of sugars is lowered.

Selecting appropriate enzyme dosages and combination is important to achieving the desired sugar yield. Niagam and Singh (1995) reported that with a careful balance of the ratio of amyloglucosidase to α–amylase, high - glucose syrup (30 – 50 % glucose, 30 – 40 % maltose) or high – maltose syrup (30 – 50 % maltose, 6 – 10 % glucose) could be achieved.

## Conclusion

Malt extract has successfully improved reducing sugar production by combining it with α-amylase and amyloglucosidase from the traditional methods of hydrolysis used in the past. The success of the combinations of malt extract with α–amylase and amyloglucosidase over other methods could be due to enzyme type, α–amylase and β-amylase present in malt extract which was able to hydrolyse α –1, 4 bonds and bypassed α –1, 6 linkages (endo-acting amylases) and hydrolyse α –1, 4 bonds and cannot bypassed α –1, 6 linkages (exo-acting amylases) respectively in amylopectin and related polysaccharides of the starch and amyloglucosidase activity which is also exo-acting enzyme and β - amylase, which rapidly break starch down starting from non-reducing end to release glucose and increase the yield of sugars.

## Conflict of interests

The authors have not declared any conflict of interest.

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African Journal of Biotechnology

*Short Communication*

# Quality characterization of Niger seed oil (*Guizotia Abyssinica Cass.*) produced in Amhara Regional State, Ethiopia

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**In the present investigation, an attempt has been made to find out cholesterol and total free fatty acid content in Niger seed oil which is the most available edible oil in Ethiopia. Acid value, peroxide value, saponification value and cholesterol content were determined. The analysis performed using Liebermann-Burchard method revealed no level of cholesterol content. Unlike the peroxide value, acid value and saponification value of Niger seed oil show high values in comparison with the maximum permissibility level of Codex Standard for Named Vegetable Oils (CODEX STAN210-1999). In conclusion, this study could be a guide line, to understand the quality of Niger seed oil in Amhara Regional State, Ethiopia. Therefore, companies producing and marketing Niger seed oil are enjoined to inform the public by labeling their products as "cholesterol free".**

**Key words:** Niger seed oil, cholesterol and free fatty acid.

## INTRODUCTION

Niger is an oilseed (Neug), which is a new source of vegetable oils; a crop that is produced from Guizotia Abyssinica. It is found mostly in the northern and central highlands at elevations between 1,800 and 2,500 m. Niger seed is the most important oil crop in Ethiopia and a minor crop in India but it is not involved in the world-wide oilseed trade. It provides 50 to 60% of Ethiopia's indigenous edible oil but only 2% of India's total oilseed production (Ramadan and Mörsel, 2002). It represents also a minor oilseed crop in some other African countries. Besides cookery, Niger seed oil can be used in the manufacture of soap, paints, or as a lubricant or illuminant. The protein-rich meal which remains after oil extraction is used as a feed, manure or fuel. Regarding the fatty acid profile, Niger seed oil resembles that of safflower and sunflower with its high content of linoleic acid (C18:2*n*–6) which may be up to 85% depending on the origin (Ramadan and Mörsel, 2002).

Ethiopian oilseeds and pulses are known for their flavor and nutritional value as they are mostly produced organically. For instance, the Ethiopian white sesame seed is used as a reference for grading in international markets(http://www.ethiopianexporters.com/products.html ). Ethiopia's major oilseed and pulse exports include sesame seeds, Niger seeds, linseeds, sunflower seeds, groundnuts, rapeseeds, castor oil seeds, pumpkin seeds,

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**Table 1.** Acid value, peroxide value, saponification value and cholesterol content of Niger seed oil sample.

| Parameter | Codex standard | Experimental value |
|---|---|---|
| Acid value (mg KOH/g) | 0.6 | 8.69 ± 0.39 |
| Peroxide value (meq.peroxide/kg) | 10 | 3.80 ± 0.28 |
| Saponification value (mg KOH/g) | 188-192 | 162.76 ± 0.69 |
| Cholesterol (mg/l) | *ND | *ND |

*ND, Not detected.

haricot beans, pea-beans, horse beans and chick peasy.

Almost every adult at present times develops some degree of atherosclerosis, commonly known as “hardening of the arteries”. Atherosclerosis leads to strokes, heart attacks and other serious health problems. High cholesterol, smoking and high blood pressure are the principal risk factors linked to heart disease.

The word “cholesterol” may quickly be associated with chronic heart disease and other heart problems. However, cholesterol also has essential functions in the body such as providing essential components of membrane and serving as a precursor of bile acids, steroid hormones and vitamin D. Consuming cholesterol in our diet increases the level of Low Density Lipoproteins (LDLs). There are different varieties of vegetable oil brands in our markets and all of them claim to be cholesterol free. Due to increasing awareness on the health implications of high cholesterol in the diets, most people now prefer to purchase cholesterol free vegetable oils (Attarde et al., 2010).

One of the other major problem in fats and oil is the development of rancidity (oxidative) and deterioration of its quality due to multiple environmental and storage conditions. The overall effect of oxidation appears in unnecessary economic loss. Oxidation is said to be off-flavor, quality, which results from reaction of atmospheric oxygen. The oxidized oils not only deteriorate the taste of foods to which they are added but are considered to create many health problems, that is, diarrhea, poor rate of growth etc. Therefore, the quality of oils is measured by determining selected quality parameters such as peroxide value, free fatty acid and color (Javid et al., 2003).

Normally, fatty acids are found in the triglyceride form; however, during processing the fatty acids may get hydrolyzed (reacts with water) into the free fatty acid. The presence of free fatty acids (FFAs) in oil is an indication of insufficient processing, lipase activity, or other hydrolytic actions (Gaye, 2009). Therefore, the present study was carried out to estimate the extent of rancidity of Niger seed oil under room temperature.

## MATERIALS AND METHODS

The non-branded Niger seed oil used for the study was bought from Bahir Dar small scale oil factory. Acid, peroxide, and saponification values were determined by standard methods (AOCS, 2003). Cholesterol content was estimated using Liebermann-Burchard reagent (Attarde et al., 2010).

## RESULTS AND DISCUSSION

### Acid value

Acid value was determined to obtain Niger seed oil total free fatty acids that are used in this study. In this study, acid value obtained was 8.69 ± 0.39 mg KOH/g (Table 1). Its value is much higher than the Codex Standard for Named Vegetable Oils (CODEX-STAN210-1999) (0.6 mg KOH/g). This hydrolysis is probably caused by a variety of agents presence, moisture in the oil, elevated temperature (above room temperature) and most important of all, lipases (enzyme) coming from the source or contaminating microorganisms. This observation supports previous study that unrefined vegetable oils had higher acid value than recommended value (Rajko et al., 2010), which indicate high free fatty acids and leads to a tendency to become rancid that is off-flavor (Tamzid et al., 2007). Probably, during processing the fatty acids may react with water and get hydrolyzed into the free fatty acids. In addition, Niger seed oil is processed other than chemical refining methods (neutralization), which lower the free fatty acid content (Cmolik and Pokorny, 2000). Long storage of the oil seeds before or after processing may also have been responsible. Kalua et al. (2008) discussed that there were changes in oil quality during cold temperature storage of the fruit. This high content of free fatty acid levels has high probability for decreasing the ability of liver to store sugars (Gur and Harwood, 1991.

### Peroxide value

Peroxide value is the chemical parameter which is frequently used. It is applied in Niger seed oil sample for the purpose of estimating the Niger seed oil oxidation towards unsaturation apart from getting estimations on the stability of Niger seed oil at low temperature. On top of that, peroxide value is closely related to certain samples’ unsaturation; the more unsaturated a sample is, the higher the probability to have high peroxide value.

According to Codex Standard for Named Vegetable Oils (CODEX-STAN210-1999), the peroxide value for Niger seed oil is 10 meq.peroxide/kg. The high values of peroxide obtained could indicate the onset of primary oxidation due to lipid degrading enzymes like peroxidase and lipoxygenase (Onyeka et al., 2005).

In this study, peroxide value obtained was 3.80 ± 0.28 meq.peroxide/kg (Table 1). Its value is lower than the codex standard permissibility level, which indicates that Niger seed oil may contain preservatives either synthetic or natural hence, reduces the high peroxide value over time. In addition, Niger seed oil may have low content of unsaturation, which are responsible for oxidation and leads to rancidity or off-flavor and present results are in line with the reported literature (Chabiri et al., 2009; Dimberu and Belete, 2011).

## Saponification value

Saponification value is determined to obtain Niger seed oil chain length that is used in this study. According to Codex Standard for Named Vegetable Oils (CODEX-STAN210-1999), saponification value of Niger seed oil is in the range of 188-192 mg KOH/g with the average value of 190 mg KOH/g. In this study, saponification value obtained is 162.76 ± 0.69 mg KOH/g (Table 1), which suggest that it contains high molecular weight long chain fatty acids hence are unsuitable for soap making and also unsuitable for human nutrition (Akinhanmi and Atasie, 2008). Lower saponification value indicates high proportion of longer fatty acids since saponification value is inversely proportional to the average molecular weight or chain length of the fatty acids (Muhammad et al., 2011). Therefore, the shorter the average chain length ($C_4$-$C_{12}$) the higher the saponification number (Tamzid et al., 2007). The value obtained for Niger seed oil during this study show that it contains high amounts of long chain fatty acids (< $C_{12}$). Geographical factor most probably owing to influence the Niger seed oil product where different plantation area gives different products (Khazainah et al., 2011).

## Cholesterol content

In this study cholesterol was not detected which is in line with the recommended value. It suggests that Niger seed may have high content of phytosterols, which has the ability to decrease cholesterol levels (Mortuza, 2006; Dept Health and Human Services, Food and Drug Administration, 2000).

Phytosterols compete with cholesterol absorption and uptake in the small intestine thereby reducing the supply of cholesterol in the blood stream (Muhammet and Samija, 2006). Since high blood total cholesterol and low-density lipoprotein (LDL) cholesterol levels are the main risk factors for coronary heart disease (CHD) and other diseases related to atherosclerosis, reducing cholesterol levels reduces the risk of CHD. Phytosterols have no effect on the levels of triacylglycerol or HDL cholesterol. Plant sterols differ from cholesterol in the presence of a methyl or ethyl group in the side chain. This difference enables plant sterols and stanols to be absorbed minimally or not at all by the intestines. Most of the ingested plant sterols pass through the gut and are excreted. Oils containing high level of polyunsaturated fatty acid are also found to inhibit the activity of hydroxymethylglutaryl-coenzymeA-reductase (HMG-CoA-reductase) which is the regulatory enzyme in cholesterol biosynthesis (Carl et al., 2009; Seddigheh et al., 2009; Ejikeme et al., 2010).

Finding from this study opposed previous work that cholesterol is present in vegetable oils, although in small proportion (Okpuzor et al., 2009; Syed et al., 2003).

## Conclusions

The results obtained from the study showed that unlike acid value, peroxide value and saponification value of Niger seed oil sample investigated were within the standard which is recommended by Codex Standard for Named Vegetable Oils (CODEX-STAN210-1999). In addition, cholesterol content of Niger seed oil was nil therefore, it is reasonable to conclude that Niger seed oil sample investigated was adequate. The results also indicate the suitability of the oil samples for domestic or industrial applications as well as export trade.

## Conflict of Interests

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

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*Full Length Research Paper*

# Molecular characterization of the plum collection [*Prunus domestica* (L.) Borkh] of the Pedagogical and Technological University of Colombia

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**Eight Random Amplified Microsatellite markers (RAMs) were used to characterize the genetic diversity found in 14 Prunus materials belonging to the deciduous collection of the Pedagogical and Technological University of Colombia. A total of 121 bands were generated: they range from nine for the GT primer to 26 for the ACA primer, and have molecular weights between 100 and 2050 Kb. At a Nei-Li similarity level of 0.75, four groups were formed, according to the characteristics of the fruit. The number of polymorphic loci varied between 8 (GT and AG) and 21 (ACA); the higher levels of heterozygosity were CA (0.43) and CT (0.41). The average value of heterozigosity for the total population was 0.35, much lower than those found in other *Prunus* species, but higher than other fruit species where RAM markers were used. Therefore, strategies must be deployed for the collections in order to increase genetic variability, such as the introduction of wild or hybrid materials. The RAM technique proved useful as a method for assessing genetic diversity in species of the *Prunus* genus.**

**Key words:** *Prunus domestica*, genetic diversity, Random Amplified Microsatellite markers (RAMs), deciduous.

## INTRODUCTION

In the last decades of the twentieth century, fruit market has been on the increase, so one can say the century is exotic fruits century, which are mostly of tropical origin. Colombia is a mega diverse country with great potential to increase fruit production. This is due to its large edaphoclimate that makes it produce fruits of different species throughout the year, from sea level of 2,800 m altitude (Toro and Tafur, 2007).

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The country (Colombia) produces plum (*Prunus domestica*), which belongs to the Rosaceae family and is a native of Europe and Asia (Fábregas, 1995). It is one of the tropical fruits in the last years, with increased market. The main producing countries are Spain, the United States, Italy, Japan, Greece and China. It is distributed in temperate regions around the world and in the mountainous tropical areas of Latin America and Africa. The main component of its fruit is water, followed by carbohydrates, where sorbitol is present. The fruits with sweet taste are consumed fresh, those with sour taste are used for the production of jams; they are also popular as ornamental garden tree due to their early flowering and the color of their flowers (INEGI, 2003).

Boyacá is one of the richest in natural resources departments, which focuses its economic activity on traditional agriculture and is a pioneer in the national production of deciduous plant. This indicates its vocation and tradition on this type of cold weather crop, over the years. The rise of the deciduous tree is due largely to development which promoted the Colombian Institute of Agrarian Reform, Incora, at the Experimental Center established in New Colon, which included the training and technology transfer to the region (Plan National Fruit, 2006).

Considering the importance of deciduous plant for agriculture region of Boyacá, GTZ program, the Faculty of Agronomy of the Pedagogical University of Colombia (UPTC) and the Technical University of Berlin developed the Colombo-German project, which established in the Experimental Farm Tunguavita in Paipa, a collection of deciduous materials imported from different thermal floors (Fisher and Torres, 1990). Subsequently, Puentes (2008) conducted an analysis of the deciduous plant from business perspective, emphasizing the peach and plum as an alternative to the Department of Boyacá.

In Colombia, the supply of new planting materials for these deciduous crops is sparse; therefore, breeding work designed to find elite materials that adapt to our edaphoclimatic conditions has not been performed. Complete information about the pedigree of these materials is not available and their morphological characteristics are not always suitable, because the cultivars and varieties closely related may show the same morphological characteristics (Stanys et al., 2012). In the case of fruit species, molecular markers are very useful because the assessment of morphological characters consumes much time and the variety expressions must be evaluated in several years. Markers can identify quickly and efficiently polymorphisms for genetic studies by increasing their efficiency in the processes of selection (Stanys et al., 2012).

In the evaluation of the characteristic of genetic and molecular level, early studies were performed on peach (*Prunus persica* L.) (Messeguer et al., 1987) and almond (Cerezo et al*.,* 1989). Subsequently, De Vicente et al. (1998), using RFLPs and Hurtado et al. (2002), using AFLPs, were able to distinguish apricot materials (*Prunus armeniaca* L.). RAPD markers have also been used for fingerprinting of *Prunus* species, such as peach (Warburton and Bliss, 1996) and almond (Bartolozzi et al., 1998). DNA profiles based on patterns of polymorphic bands, such as RAPD (Shimada et al., 1999) and AFLP (Goulao et al., 2001) have been used to analyze the genetic variability of Japanese plum cultivars.

However, Microsatellite markers are the preferred technique used for genetic relationships studies between species and for assays of genetic diversity among cultivated species (Gupta et al*.,* 1996). This is due to high polymorphism and abundant and co-dominant inheritance. The majority of SSRs used for fingerprinting have been developed in Peach and sweet cherry (Clarke and Tobutt, 2003) and used for molecular characterization and genetic similarity of genotypes in several species of *Prunus* including peach (Aranzana et al., 2010). Microsatellite markers are also used for genetic map of peach (Aranzana et al., 2003), almond (Bliss et al., 2002), and apricot (Hurtado et al., 2002). Another application for these sequences of microsatellites is in the study of markers associated with characteristic of interest that may be included within the strategies of Marker Assisted Selection (MAS) (Testolin, 2000).

Among the Simple Sequence Repeat markers, we find that Random Amplified Microsatellite (RAMs) are very useful for measuring genetic diversity in plants and animals, difference between families, between and within species (Muñoz et al., 2008), show the basis of variation of individuals, allow you to select specific regions within the DNA molecule for determined studies, the number of polymorphisms detectable is theoretically unlimited and it is possible to analyze both information that is expressed (Henríquez, 2000). This methodology is feasible for small laboratories in terms of equipment and facilities cost, does not require prior knowledge of sequences and the use of radioactive isotopes (Hantula et al., 1996).

Markers achieved by RAMs can be used for population studies (Hantula et al., 1996). This technique is useful in identifying duplicates in banks or collections of germplasm, used for the establishment of phylogenetic relationships in different fruit species (Bonilla et al., 2008; Sanabria et al., 2006). In this context, this research aims to identify the genetic variability present in the collection of plum (*Prunus domestica*) of the University Pedagogical and Technology of Colombia, to establish genetic relationship that exists between the materials and thereby provide a tool that can be used as breeding strategies on the species and to identify elite material that may be a new productive alternative for our farmers.

## MATERIALS AND METHODS

A total of 11 accessions of plum (2 of apricot- *Prunus armeniaca* and 1 of almond- *Prunus dulcis*), belonging to deciduous genebank

**Table 1.** *Prunus* materials of the deciduous collection from the UPTC used for the assessment of genetic diversity with Random Amplified Microsatellites (RAMs).

| S/N | Material | Classification** |
|---|---|---|
| 1 | Horvin | Red Plum Var. |
| 2 | Chileno | |
| 3 | Methey | Red Plum Var. |
| 4 | Morado | |
| 5 | Reina Claudia | |
| 6 | Gold Fruly | . |
| 7 | Early | |
| 8 | Real Beauty | Red Plum Var. |
| 9 | Beauty | Red Plum Var. |
| 10 | Ecuatoriano | Yellow Plum Var. |
| 11 | Chileno-2 | |
| 12 | Albaricoque Bulida | |
| 13 | Albaricoque Canino | |
| 14 | Almendro. | |

*Source: Martínez (2013).

**Table 2.** Primers used in the RAM Microsatellite technique.

| Markers | Sequence (5´to 3´) |
|---|---|
| CCA | $DDB(CCA)_5$ |
| CGA | $DHB(CGA)_5$ |
| ACA | $BDB(ACA)_5$ |
| GT | $VHV(GT)_5G$ |
| AG | $HBH(AG)_7A$ |
| CT | $DYD(CT)_7C$ |
| TG | $HVH(TG)_7T$ |
| CA | $DBDA(CA)_7$ |

of the UPTC and established on the experimental farm at Tunguavita, Paipa were evaluated (Table 1).

#### Molecular characterization

Molecular characterization was done in Molecular Biology Research Laboratories, Gebimol and Bioplasma, of UPTC, Colombia, Tunja. For DNA extraction, Dellaporta et al. (1983)'s protocol was used. The total DNA was visualized with 0.8% agarose gels, stained with Z-Vision, in a Maxicell EC-340 Primo Gel Electrophoresis System chamber. In order to determine the DNA concentration of each accession, a dilution curve of DNA from bacteriophage Lambda with an initial concentration of 20 ng/µL was made. For analysis, eight RAM primers synthesized by Technologies Inc. were used (Table 2). The amplification reaction RAMs was prepared in a sterile microcentrifuge tube (1.5 ml) to a final volume of 25 µL. The reaction mixture was prepared with 1X buffer, 1.5 mM $MgCl_2$, 0.2 M dNTPs, 1U Taq Polymerase, 2 uM primer and 10 ng genomic DNA (Sanabria et al., 2006).

The following designations are used for degenerated sites: H (A/T/C); B (G/T/C); V (G/A/C) and D (G/A/T).

The amplification was carried out in a thermocycler PTC 100 Programmable Thermal Controller (MJ. Research, Inc). Initial denaturation was at 95°C for 5 min; denaturation at 95°C for 30 s, annealing temperature of 50°C (AG and CA primers), 55°C (CCA, TG and CT primers) and 58°C (GT and CGA primers) for 45 s, an extension of 72°C for 2 min, 37 cycles of denaturation, and, finally, extension at 72°C for 7 min. Amplified products were separated by electrophoresis in polyacrylamide gels in a ratio of 37:1 (acrylamide: bisacrylamide) at 7% and 150 V for 1 h in a small DNA Sequencing System chamber, Fisher Biotech FB-SEQ-3545. The staining was carried out using silver salts (Sanabria et al*.,* 2006).

#### Statistical analysis

An absent (zero) and present (one) binary matrix was generated. The genetic similarity between individuals was calculated using the similarity coefficient of Nei and Li (1979), also known as DICE (1945) (Sneath and Sokal, 1973). The cluster analysis was conducted by the UPGMA method and a dendrogram was generated using the statistical package NTSYS (Numerical Taxonomy System for Personal Computer, PC version 2.02). To evaluate the genetic diversity, unbiased heterozygosity and percentage of polymorphic loci were estimated using the statistical package TFPGA (Tools For Population Genetic analysis, version 1.3, 1997). Unbiased statistical f with a confidence interval of 95% was determined.

## RESULTS AND DISCUSSION

In the analysis with the Nei-Li coefficient, at a similarity level of 0.75, the population was distinguished into four groups based mainly on the characteristics of the fruit (Figure 1). At a similarity level of 0.65, we found Horvin and Methey plums in group I, according to the classification of Campos (1989); they belong to the red varieties, which are characterized by a high pruina content, rounded shape, small size and average weight of 35 g. Horvin differs from Methey, basically, at the plant level (Campos, 2013).

In the second group, we found Red Beauty and Beauty sharing a similarity of 0.80 and a litter further away (0.75) was the Early plum. Red Beauty is the earliest in the Spanish market; Beauty has a red skin with slight tips, pruina, an insipid, slightly acidic taste (Carrera, 2002), and a heart-shaped medium size with an average weight of 70 g. Within this group, the Ecuadorian and Chilean-2 materials share a similarity of 0.80; they are farther away from the rest of the plums in this group. The Ecuadorian variety is characterized by having yellow skin, with reddish dyes at maturation and low contents of pruina (Campos, 2013). In group II, a similarity of 0.85 is seen for Gold Fruly and Queen Claudia. The last one, with a European origin, has yellowish-green skin; it is heart-shaped and large in size; its production is very prolific. The yellow Japanese variety (Ogden) has yellow flesh; its juicy, pruina, has a heart-shape, large size, and also known by the common name, egg yolk. It is wrongly called Queen Claudia because its characteristics correspond to a yellowish-green skin, yellow stoneless pulp (Campos, 2013).

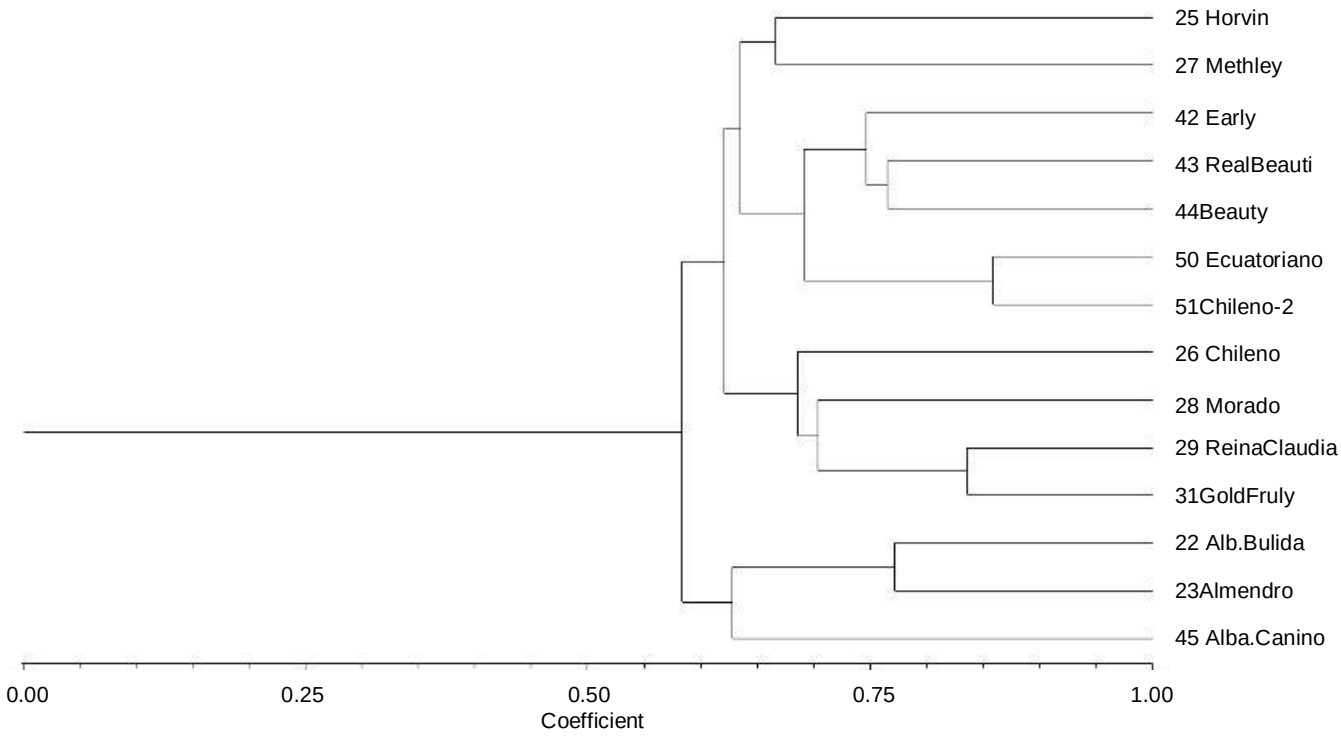


**Figure 1.** Dendrogram of the 11 *Prunus domestica* materials, based on the Nei-Li similarity coefficient and calculated with eight RAM markers with the UPGMA classification method, SAHN and TREE of NTSYS-pc version 1.8 (Exeter Software, NY, USA).

In the fourth group, we found the apricot (*Prunus armeniaca* L.). Among the most important varieties are Bulida (of Spanish origin, adapts to all kinds of soils and very juicy) and Canina, which has good quality fruit, marketing (Martínez et al., 2004) and presents the lowest similarity with the rest of the evaluated materials. The almond tree is a rustic species that adapts to extreme conditions, such as dry climates. It is supported by temperate climates; therefore, flowering is early and requires little cold. Its affinity with the apricot is very high (Gallego, 2010), which can be visualized in the dendrogram.

The analysis of the similarity between the materials from the evaluated deciduous collection showed high homogeneity, with associations responding to morphological characteristics of varieties, specially related to fruit (Stanys et al., 2008). In addition, it was established that the materials that are believed to be the same (Chileno and Chileno-2) were found to be two different materials from Chile. We cannot establish whether the groups are due to the geographical site where the materials were collected because there is no such information. Finally, compatibility between the plum and almond and other species related to the almond or apricot can be observed.

The eight RAM Microsatellite markers used for the assessment of the genetic diversity in the plum generated a total of 121 bands, which ranged from 9 for the ACA primer to 26 for the GT primer, with molecular weights between 100 and 1000 kb. The number of polymorphic loci ranged from 5 to 16 for GT and CGA, respectively (Table 3). The number of bands was considered adequate for estimating genetic parameters (Stanys et al*.,* 2012). The CCA primer made the highest contribution to the observed genetic variation, Fst 0.85. This means they can be useful for achieving greater differentiation between materials of the *Prunus* genus.

Heterozygosity values ranged between 0.28 (CGA) and 0.43(CA). The average value of heterozygosity for the general population of 0.35 was much lower than that reported in other SSR studies for *Prunus* species; they include work done on peaches (*Prunus persica* L.) by Aranzana et al. (2010) (0.46); almond by Turkoglu et al. (2010) (0.61) and Fernández et al. (2009) (0.72); sweet cherry by Fathi et al. (2008) (0.79) and Wünsch et al. (2002) (0.49); apricot by Schueler et al. (2003) (0.66); guava using RAM markers by Martín et al. (2011) (0.70-0.58) and (Sanabria et al*.,* 2006). In other fruit species where genetic diversity was studied with RAM markers, there were expected average heterozygosity values lower than those found in this study (Bonilla et al. (2008); they were Cape gooseberry (0.25) and mandarin (0.31) (Mora et al., 2013).

In the plum, Carrasco et al. (2012) molecularly

**Table 3.** Estimated average heterozygosity (He) and percentage of polymorphic loci for the eight RAM primers evaluated in 11 plum materials, 2 apricot materials and 1 almond material.

| Primer | N° Polimorphic loci | He estimated | % Loci Polimorphic (95%) | Fst | SD |
|---|---|---|---|---|---|
| ACA | 21 | 0.33 | 80.8 | 0.48 | 0.06 |
| TG | 11 | 0.35 | 100 | 0.60 | 0.09 |
| CGA | 18 | 0.28 | 85.7 | 0.61 | 0.09 |
| CT | 13 | 0.41 | 92.8 | 0.51 | 0.06 |
| CCA | 11 | 0.34 | 84.61 | 0.61 | 0.07 |
| CA | 14 | 0.43 | 93.33 | 0.37 | 0.06 |
| AG | 8 | 0.34 | 66.67 | 0.46 | 0.09 |
| GT | 8 | 0.37 | 88.8 | 0.42 | 0.09 |
| Population total | | **0.35** | **86.4** | **0.51** | **0.03** |

characterized cultivars of the Japanese plum (*Prunus salicina*), using the molecular markers SSR and ISSR. The mean values of observed and expected heterozygosity for the SSRs were 0.9 and 0.8. These results suggest that the high level of genetic variability can be explained by self-incompatibility mechanisms favoring exchanges between genetically distant cultivars of *Prunus* strategies and intra- and interspecific hybridization, frequently used in breeding programs for plums. Ahmad et al. (2004) studied genetic diversity in 20 cultivars of plums using simple sequence repeats. They obtained heterozygosity values of 0.70. Mnejja et al. (2004), in eight plum cultivars, found heterozygosity values of 0.73; thereby, showing great variability in the material being evaluated. This does not agree with the results obtained in this study. it can be due to the selected number and class, such as the marker type materials used. Shimada et al. (1999) studied 42 cultivars of Japanese plum using RAPD markers and found low levels of polymorphism (24%). Goulao et al. (2001) analyzed 28 plum cultivars using ISSR and AFLP, and again, the genetic variability (polymorphic ISSR = 87.4% and 62.8% polymorphic AFLP) was less than that found in this study.

Aran et al. (2012) morphologically and molecularly differentiated selected plum seedlings with 22 RAPD primers to improve patterns. They found 195 polymorphic bands and a similarity between 0.27 and 0.77. Studying the genetic diversity in 27 plum cultivars, using 10 RAPD primers, Hend et al. (2009) obtained 97.3% polymorphism and a genetic similarity between 0.18 and 0.80. Shimada et al., (1999) also studied the genetic variation of plum cultivars with RAPD markers and reported 24% polymorphism. It should be noted that Shimada studied commercial genotypes while the samples used in Aran et al. (2012)'s and Hend et al. (2009)'s studies were wild materials. The diversity among wild genotypes compared with a commercial one can be addressed by the narrow genetic diversity due to selection within the cultivated materials. Many studies have been conducted on plum cultivars with important results obtained using RAPDs (Liu et al., 2007, Lisek et al., 2007).

However, the value of average heterozygosity found in this study (0.35) was higher than that reported in studies characterizing the genetic diversity of natural populations of *Prunus davidiana* (0.17) (Cheng et al., 2011) and peach (0.22) (Martinez et al. 2003). Furthermore, the values were greater than the average estimated genetic variability of allogamous plants (He = 0.22 for dominant markers; Nybom, 2004). A low level of polymorphism in the sweet cherry has also been detected by Gerlach and Stösser (1997), using RAPD markers. This probably reflects a narrow genetic base in the germplasm of the sweet cherry and peach, but is consistent with the assertions of Imbert and Lefévre (2003), who believe that an excess of heterozygosity is not very usual in tree species and generally this is favored by self-incompatibility and interspecific crosses. The narrow genetic base used to develop cultivars and the existence of common ancestors in the pedigree of peaches and plums could explain the results found (Okie and Hancock, 2008).

The coefficient of genetic differentiation (Fst) obtained in evaluating 11 plum materials with eight RAM markers was 0.51 with a standard deviation of 0.03 (Table 3). According to Wright (1978), values of 0.25 show high genetic differentiation, which may be reflected in the high degree of domestication that these materials have suffered, since most of them are commercial varieties. Carrasco et al., 2012 found an index fixation of F = -0.127, higher than those in other allogamous species such as the almond (F = 0.15; Fathi et al., 2008), and wild apple (F = 0.10; Coart et al., 2003). Excess heterozygosity could be explained by the negative selective mating related to the self-incompatibility system, such that the parental lines carry different favored alleles than the interspecific cross usually used in plum breeding

programs.

The low values of heterozygosity that were seen corroborate with high values of identity and Nei-li genetic distances (1978, 1979) (0-65-0.79). The analysis of molecular variance showed that 73% of the observed genetic variability is located within the formed groups and only 27% is due to differences between the groups; which suggests lower and finer levels of hierarchy than those used in this study. Casas et al. (1999) also reported high values of similarity using RAPD markers (0.71). In the last decade, molecular markers have become a valuable tool that provides a huge amount of genetic information on fruit crops, allowing its use for the identification of genotypes and the evaluation of the genetic diversity of this species group. Simple sequence repeats (SSRs) and random amplified (RAMs) microsatellites can be valuable tools for the study of genetic diversity and conservation, especially for cultivars or genotypes of interest before they disappear. On the other hand, in the case of cultivated species of this genus, some genetic limitation has been described due to the use of a limited number of varieties in breeding programs. The introduction of genes from these wild Prunus species through interspecific breeding programs could be an interesting addition to improve existing programs by widening the genetic basis and increasing the efficiency in the selection process (Scorza et al., 1996).

## Conclusion

Molecular characterization of the plum collection of the Pedagogical University of Colombia with RAM markers showed low genetic diversity. It needs to be developed through the incorporation of wild materials or interspecific hybrids that would contribute new allelic variants to the collection. The RAM technique proved to be a useful tool for the differentiation of species materials of the *Prunus* genus.

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*Full Length Research Paper*

# Genetic assessment of some phenotypic variants of rice (*Oryza* spp.) for some quantative characters under the Gangatic plains of West Bengal

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**Twenty two (22) recombinant inbreed lines (RIL's) derived from interspecific cross derivative of *Oryza sativa* and *Oryza rufipogon* along with two local check varieties MTU 7029 and Ranjit were evaluated in randomized block design (RBD) with two replications at two different environments (1st at Regional Research Station, New Alluvial Zone (NAZ), Bidhan Chandra Krishi Viswavidyalaya, Sub-Centre, Chakdah, Nadia, West Bengal during Kharif season 2009 and 2nd at Instructional Farm, Bidhan Chandra Krishi Viswavidyalaya, Jaguli, Nadia, during Kharif season 2010), to study the polygenic variations in yield for yield and its attributing characters and their cause and effect relationship. The analysis of variances revealed the significant differences among the 24 genotypes against all the characters except panicle weight, grain length, grain breadth and grain L/B ratio. The magnitude of phenotypic coefficients of variation (PCV) was higher than genotypic coefficients of variation (GCV) for all the characters suggesting the influences of the environmental forces on the expression of these characters. High PCV and GCV values were observed in grain yield per plant, 1000 grain weight, L/B ratio, grain breadth and panicle weight. High heritability coupled with moderate to high genetic advance as percent of mean for plant height, panicle weight, grain length, grain L/B ratio, 1000 grain weight and yield per plant while low heritability estimates along with low genetic advance were observed for fertility percentage and florets number per panicle. In general, genotypic correlation coefficients were higher than their corresponding phenotypic correlation coefficients. Path coefficient analysis revealed that number of characters chosen was very much appropriate as evident from low value of residual effect. Maximum positive direct effect was imparted by number of gains per panicle followed by grain L/B ratio, days to 50% flowering and panicle length respectively. Florets number per panicle imparted the maximum negative direct effect followed by grain breadth, fertility percentage and panicle number per plant. Per se performance revealed that two lines viz; KS-7 and KS-13 were promising in respect of grain yield and some other yield related traits. Number of grains per panicle and floret number per panicle give significant positive correlation with yield.**

**Key words:** Correlation, genotypic coefficients of variation, genetic advance, heritability, phenotypic coefficients of variation, rice, recombinant inbreed lines.

## INTRODUCTION

Worldwide, rice (*Oryza sativa* L.) is the second most important cereal crop, just after wheat. It provides 20% of the world's dietary energy supply (FAO, 2004)). Since grain yield is a complex trait, indirect selection through correlated, less complex and easier measurable traits would be an advisable strategy to increase the grain yield. Efficiency of indirect selection depends on the magnitude of correlations between yield and target yield

components (Singh and Chaudhary, 1977; Fakorede and Opeke, 1985; Toker and Cagirgan, 2004; Bhatti et al.2005).

Breeding strategy in rice mainly depends upon the degree of associated characters as well as its magnitude and nature of variation (Zahid et al., 2006; Prasad et al., 2001). Path coefficient analysis furnishes information of influence of each contributing traits to yield directly as well as indirectly and also enables breeders to rank the genetic attributes according to their contribution (Cyprien and Kumar, 2011). In rice, selection of high yielding cultivars via specific traits requires knowledge of not only final yield but also the many compensation mechanisms among yield components resulting from changing genotypic, environmental and management factors. Grain yield of rice is a quantitative trait which is controlled by many genetic as well as environmental factors (Singh and Singh, 2004; Ceyhan and Avci, 2005; Ranjan et al., 2006). For selection in rice, information on correlation coefficient always has been helpful as a basis for selection in a breeding program (Akhtar et al., 2011). Path coefficient analysis partitions this relationship into direct and indirect matrix presenting correlation in a more meaningful way (Mohsin et al., 2009). The path analysis has been used by plant breeders (Indu Rani et al., 2008; Togay et al., 2008; Ali et al., 2009) to support in identifying traits that are promising as selection criteria to improve crop yield and to detect the amount of direct and indirect influence of the causal components on the effect component (Bhatti et al., 2005).

Path coefficient analyses are particularly useful for the study of cause-and-effect relationships because they simultaneously consider several variables in the data set to obtain the coefficients. Determination of correlation and path coefficients between yield and yield criteria is important for the selection of promising rice genotypes to be used in any meaningful breeding program. In this context, the present studies has been made to assess the variability and cause effect relationship with grain yield per plant in two different environment and pooled analysis has been done over the two environments to study the polygenic variations in yield for yield and its attributing characters and their cause and effect relationship.

## MATERIALS AND METHODS

The experimental material consisted of 22 stable recombinant inbred lines (RIL's) developed from an interspecific cross *O. sativa* (var, B-32 Selection 4) /*Oryza rufipogon* (var, B-127). Finally, after 11 genes rating of selection, 22 stable RIL's were established. They were designated as Kakdwip selection-1 to Kakdwip selection-22. These were studied during Kharif 2010 along with two local check varieties MTU 7029 and Ranjit at two different environment; one at Regional Research Station, New Alluvial Zone, Sub-Centre Chakadaha (Latitude 23° 30′ N, longitude 89°E and altitude 9.75 m above mean sea level) and another at Instructional farm (Latitude 22° 93′ N, longitude 88.59° E and altitude 9.75 m above mean sea level), Jaguli of Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal, India, under rainfed shallow lowland condition. The soil of the 1$^{st}$ experimental site was sandy loam in texture, normal in pH (7.00), good water holding capacity and high fertility status while the 2$^{nd}$ experimental site was sandy loam in texture, normal in pH, good water holding capacity and medium fertility status. The experiments were laid out in Randomized Block Design (RBD) with two replications having a plot size of 5 × 2 m against each entry. Single seedling per hill was transplanted manually for each entry maintaining a spacing of plant of 20 cm × 15 cm between lines and between plants within lines respectively. Normal agronomic practices were followed to obtain a good harvest. Observations were recorded on five plants selected randomly from each entry against 14 quantitative characters such as plant height (cm), days to 50% flowering, days to maturity, number of panicle per plant, panicle weight (g), panicle length (cm), florets number per panicle, number of grains per panicle, percentage fertility, grain length (mm), grain breadth (mm), grain L/B ratio, 1000 grain weight (g), grain yield per plant (g). Mean data pooled over two environments were statistically analyzed using appropriate computerized statistical programme for the estimation of variability, heritability, genetic advance and correlation coefficient and path coefficient analysis (Deway and Lu, 1959) among the tested characters.

## RESULTS AND DISCUSSION

The mean performance for 14 quantitative characters of 22 RIL's and checks varieties in environment 1, environment 11 and pooled data over the two environments are presented in Table 1, 2 and 3, respectively. All the lines exhibited considerable variability for all the characters studied. Line KS-22 recorded highest plant height (135.70 cm) followed by lines KS-13 and KS-20 while check variety MTU-7029 (101.65 cm) showed lowest plant height followed by lines KS-8, KS-2 and KS-6 respectively in environment 1. In environment 2, line KS-17 recorded maximum plant height (145.90 cm) followed by lines KS-20, KS-19 and KS-22 while check variety Ranjit showed the shortest plant height (112.70 cm) followed by lines KS-8, KS-5 and KS-4 respectively. Pooled performance over two environments showed that line KS-22 was the tallest (139.77cm) followed by lines KS-20 and KS-13 while check variety Ranjit showed minimum plant height (108.10 cm) followed by variety MTU-7029 and line KS-8, respectively.

In environment 1, KS-12 was last in days to 50% flowering (123.00 DAS) followed by lines KS-21, KS-10 and KS-11 respectively while line KS-4 was first in days to 50% flowering (111.00 DAS) followed by KS-7, KS-17

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**Table 1.** Mean performance of 22 RIL's of rice along with check varieties for 14 characters during Kharif 2009 (Environment-1).

| Genotype | Characters | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Plant height (cm) | Days to 50 % flowering | Days to maturity | No. of panicle/plant | Panicle weight (g) | Panicle length (cm) | Florets No./ panicle | No. of grains/ panicle | Fertility % | Grain length (mm) | Grain breadth (mm) | Grain L/B ratio | 1000 grain weight(g) | Grain yield/ plant (g) |
| KS-1 | 111.50 | 112.50 | 143.50 | 11.00 | 3.87 | 22.23 | 181.15 | 138.10 | 75.91 | 8.27 | 3.09 | 2.68 | 21.55 | 25.50 |
| KS-2 | 104.25 | 118.00 | 147.00 | 13.00 | 2.27 | 21.27 | 142.85 | 107.15 | 75.03 | 8.39 | 3.03 | 2.76 | 17.97 | 24.53 |
| KS-3 | 113.25 | 115.00 | 149.50 | 11.00 | 2.38 | 25.08 | 183.05 | 122.75 | 67.11 | 8.71 | 3.06 | 2.89 | 19.50 | 20.04 |
| KS-4 | 108.80 | 111.00 | 145.00 | 11.50 | 2.08 | 21.21 | 172.90 | 110.70 | 64.55 | 8.15 | 2.80 | 2.90 | 14.40 | 18.97 |
| KS-5 | 114.00 | 116.00 | 145.00 | 10.50 | 2.72 | 21.77 | 135.80 | 100.10 | 73.59 | 8.59 | 3.10 | 2.76 | 24.99 | 17.72 |
| KS-6 | 104.75 | 112.50 | 148.00 | 10.50 | 2.70 | 20.03 | 139.65 | 103.10 | 73.95 | 8.05 | 2.32 | 3.47 | 14.85 | 16.70 |
| KS-7 | 113.00 | 111.50 | 148.50 | 12.00 | 2.69 | 23.75 | 157.00 | 101.00 | 64.22 | 8.23 | 3.21 | 2.56 | 23.81 | 30.28 |
| KS-8 | 104.05 | 116.50 | 149.00 | 13.00 | 3.33 | 22.51 | 163.80 | 116.30 | 70.92 | 8.54 | 3.11 | 2.74 | 21.16 | 17.55 |
| KS-9 | 110.50 | 114.00 | 144.00 | 12.00 | 3.27 | 22.43 | 164.90 | 139.55 | 85.95 | 8.03 | 2.88 | 2.68 | 22.52 | 21.81 |
| KS-10 | 113.05 | 122.00 | 152.00 | 11.00 | 2.10 | 22.01 | 134.05 | 100.65 | 74.68 | 9.59 | 2.37 | 4.04 | 22.87 | 19.29 |
| KS-11 | 117.45 | 121.50 | 150.00 | 11.50 | 2.82 | 21.57 | 114.45 | 87.85 | 76.58 | 9.82 | 2.24 | 4.38 | 26.87 | 19.01 |
| KS-12 | 109.65 | 123.00 | 151.50 | 11.00 | 4.23 | 21.70 | 179.75 | 109.35 | 61.10 | 8.33 | 2.39 | 3.34 | 25.58 | 19.70 |
| KS-13 | 129.45 | 120.50 | 150.50 | 14.00 | 2.35 | 19.36 | 142.35 | 108.75 | 76.49 | 7.78 | 2.21 | 3.51 | 18.21 | 28.21 |
| KS-14 | 108.80 | 117.50 | 147.00 | 11.00 | 3.27 | 21.44 | 160.65 | 114.55 | 71.07 | 8.03 | 2.39 | 3.35 | 22.75 | 24.04 |
| KS-15 | 108.20 | 116.00 | 149.50 | 15.00 | 2.91 | 22.69 | 145.70 | 112.80 | 77.56 | 8.20 | 2.83 | 2.89 | 20.88 | 20.69 |
| KS-16 | 112.95 | 113.50 | 143.00 | 12.50 | 2.60 | 23.24 | 162.05 | 115.25 | 72.45 | 7.90 | 2.66 | 2.86 | 21.40 | 24.94 |
| KS-17 | 110.45 | 112.00 | 144.50 | 11.50 | 2.41 | 22.51 | 155.10 | 112.65 | 72.62 | 8.19 | 2.21 | 3.70 | 19.21 | 18.48 |
| KS-18 | 111.70 | 114.00 | 143.50 | 14.50 | 2.94 | 22.37 | 151.20 | 106.75 | 71.53 | 8.19 | 2.66 | 3.07 | 18.25 | 15.13 |
| KS-19 | 108.75 | 115.00 | 142.00 | 12.00 | 2.54 | 23.43 | 166.45 | 115.45 | 69.50 | 8.45 | 2.37 | 3.57 | 19.23 | 20.08 |
| KS-20 | 127.90 | 119.00 | 151.00 | 11.50 | 2.73 | 20.24 | 127.55 | 93.20 | 73.59 | 10.03 | 2.46 | 4.09 | 31.45 | 19.29 |
| KS-21 | 123.30 | 122.00 | 151.00 | 13.00 | 2.63 | 23.02 | 177.35 | 126.05 | 71.50 | 8.30 | 2.53 | 3.28 | 19.15 | 28.09 |
| KS-22 | 135.70 | 119.00 | 152.00 | 11.50 | 3.25 | 22.10 | 138.45 | 88.90 | 65.23 | 10.06 | 2.58 | 3.89 | 22.83 | 15.86 |
| Ranjit* | 103.50 | 115.50 | 144.50 | 12.00 | 2.80 | 21.96 | 175.30 | 109.65 | 62.57 | 7.97 | 2.16 | 3.67 | 14.79 | 19.22 |
| MTU 7029* | 101.65 | 115.00 | 149.50 | 14.00 | 2.35 | 21.43 | 151.20 | 105.45 | 70.11 | 7.86 | 2.61 | 3.00 | 18.54 | 19.94 |
| Mean | 112.76 | 116.35 | 147.56 | 12.10 | 2.81 | 22.05 | 155.11 | 110.25 | 71.56 | 8.45 | 2.63 | 3.25 | 20.95 | 21.04 |
| CD (0.05) | 2.35 | 2.69 | 2.28 | 2.79 | - | 1.39 | 41.57 | 24.16 | 11.66 | - | - | - | 1.21 | 3.37 |

*Check variety.

and KS-1. In case of environment 2, KS-12 ranked last in days to 50% flowering (130.00 DAS) followed by lines KS-10 and KS-11 while line KS-1 was the earliest in days to 50 % flowering (117.00 DAS) followed by lines KS-4 and KS-7 respectively. Nearly similar result was obtained for the pooled data in this regards. In environment 1, KS-19 matured earliest (142.00 DAS) followed by KS-18, KS-1 and KS-9 while KS-1, KS-22(152.00 DAS), KS-21 and KS-20 were in the late maturing group. In case of environment 2, line KS-1 matured earliest (147.50 DAS) followed by lines KS-19 and Ranjit while lines KS-11, KS-13 (155.00 DAS), KS- 22 and KS-6 were very late in maturity. According to pooled data, lines KS-19 (147.25 DAS), KS-1, KS-16 and variety Ranjit were earlier than the rest of the genotypes. KS-15 recorded maximum panicle numbers per plant (15.00) followed by lines KS-18 and KS-13 while KS-5 and KS-6 had lowest panicles per plant (10.50) followed by lines KS-3

**Table 2.** Mean performance of 22 RIL's of rice along with check varieties for 14 characters during Kharif 2010 (Environment-2).

| Genotype | Characters | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Plant height (cm) | Days to 50 % flowering | Days to maturity | No. of panicle/plant | Panicle weight (g) | Panicle length (cm) | Florets No. / panicle | No. of grains/ panicle | Fertility % | Grain length (mm) | Grain breadth (mm) | Grain L/B ratio | 1000 grain weight(g) | Grain yield/ plant (g) |
| KS-1 | 132.10 | 117.00 | 147.50 | 11.00 | 5.06 | 25.38 | 257.15 | 191.25 | 74.66 | 8.00 | 2.95 | 2.70 | 21.90 | 29.45 |
| KS-2 | 135.00 | 120.50 | 149.50 | 15.50 | 3.05 | 24.54 | 191.70 | 117.60 | 53.64 | 7.97 | 2.79 | 2.87 | 20.00 | 32.04 |
| KS-3 | 138.25 | 119.00 | 151.00 | 15.50 | 3.72 | 28.20 | 212.85 | 161.35 | 64.25 | 8.27 | 2.61 | 3.16 | 20.07 | 35.89 |
| KS-4 | 124.65 | 118.00 | 150.50 | 16.50 | 4.69 | 26.87 | 223.00 | 170.25 | 74.12 | 8.10 | 2.78 | 2.91 | 19.12 | 36.24 |
| KS-5 | 123.85 | 123.00 | 151.50 | 11.50 | 3.22 | 23.95 | 182.00 | 107.70 | 59.21 | 7.91 | 2.94 | 2.69 | 24.29 | 28.03 |
| KS-6 | 125.00 | 119.00 | 153.00 | 20.50 | 3.65 | 23.94 | 233.65 | 132.10 | 59.78 | 7.86 | 2.68 | 2.95 | 19.90 | 32.43 |
| KS-7 | 125.80 | 118.00 | 150.00 | 20.00 | 5.23 | 26.55 | 227.50 | 145.80 | 63.76 | 8.03 | 3.07 | 2.59 | 25.25 | 35.91 |
| KS-8 | 123.15 | 124.50 | 151.00 | 13.00 | 4.07 | 24.43 | 220.80 | 151.00 | 68.34 | 7.79 | 2.99 | 2.60 | 21.68 | 32.05 |
| KS-9 | 126.30 | 121.00 | 150.00 | 14.00 | 4.20 | 24.86 | 196.30 | 130.50 | 66.27 | 8.04 | 3.00 | 2.68 | 23.67 | 31.53 |
| KS-10 | 135.65 | 129.50 | 152.50 | 15.50 | 3.31 | 25.86 | 164.60 | 110.70 | 62.76 | 9.70 | 2.86 | 3.39 | 26.30 | 35.51 |
| KS-11 | 143.05 | 129.50 | 155.00 | 13.00 | 5.19 | 27.13 | 203.55 | 128.60 | 63.13 | 9.75 | 2.84 | 3.43 | 29.90 | 32.22 |
| KS-12 | 137.15 | 130.00 | 150.00 | 11.00 | 3.14 | 26.36 | 255.40 | 147.90 | 58.43 | 9.16 | 3.01 | 3.04 | 29.01 | 27.87 |
| KS-13 | 141.85 | 126.50 | 155.00 | 12.50 | 4.48 | 22.88 | 237.65 | 179.15 | 74.47 | 8.14 | 2.96 | 2.74 | 22.02 | 33.61 |
| KS-14 | 131.60 | 122.50 | 150.50 | 15.00 | 2.79 | 25.59 | 206.90 | 145.95 | 70.25 | 8.09 | 2.99 | 2.68 | 22.12 | 26.87 |
| KS-15 | 140.70 | 119.00 | 153..50 | 13.50 | 3.39 | 23.45 | 199.50 | 128.00 | 63.89 | 7.98 | 2.99 | 2.66 | 23.46 | 34.70 |
| KS-16 | 142.15 | 120.00 | 150.00 | 14.00 | 2.45 | 26.19 | 203.20 | 156.60 | 77.05 | 7.97 | 2.94 | 2.71 | 22.39 | 29.06 |
| KS-17 | 145.90 | 119.00 | 150.50 | 13.50 | 2.58 | 25.54 | 173.40 | 120.85 | 71.27 | 7.94 | 2.48 | 3.19 | 20.31 | 26.68 |
| KS-18 | 141.60 | 120.00 | 150.50 | 17.50 | 3.88 | 26.55 | 234.20 | 159.10 | 67.80 | 7.99 | 2.81 | 2.84 | 20.00 | 35.72 |
| KS-19 | 144.60 | 119.00 | 148.50 | 14.00 | 3.24 | 25.64 | 197.50 | 133.75 | 69.61 | 8.12 | 2.77 | 2.94 | 21.72 | 37.11 |
| KS-20 | 145.00 | 125.00 | 153.00 | 20.00 | 3.04 | 24.37 | 149.85 | 73.80 | 40.04 | 9.79 | 2.91 | 3.36 | 28.82 | 24.46 |
| KS-21 | 127.25 | 124.00 | 151.50 | 12.50 | 2.35 | 24.06 | 131.70 | 102.80 | 72.71 | 8.20 | 2.43 | 3.39 | 21.61 | 22.29 |
| KS-22 | 143.85 | 126.00 | 154.50 | 23.00 | 3.18 | 24.70 | 190.55 | 117.50 | 52.36 | 10.13 | 2.86 | 3.53 | 29.66 | 34.45 |
| Ranji*t | 112.70 | 119.00 | 149.50 | 13.00 | 3.16 | 24.55 | 296.40 | 162.75 | 47.56 | 8.07 | 2.65 | 3.04 | 18.97 | 34.20 |
| MTU 7029* | 123.10 | 119.50 | 152.00 | 14.50 | 2.80 | 25.06 | 203.20 | 141.60 | 62.21 | 7.72 | 2.68 | 2.89 | 19.75 | 29.75 |
| Mean | 133.76 | 122.04 | 151.31 | 15.00 | 3.58 | 25.27 | 208.02 | 138.19 | 64.06 | 8.36 | 2.83 | 2.95 | 22.99 | 33.63 |
| CD (0.05) | 1.83 | 2.53 | 2.62 | 2.55 | - | 0.82 | 50.10 | 31.97 | 18.67 | - | - | - | 2.63 | 4.28 |

*Check variety.

and KS-1 in environment 1.

In environment 2, line KS- 22 recorded maximum panicle number per plant (23.00) followed by lines KS-6, KS-20 and KS-7 respectively while line KS-1 and KS 12 showed minimum number of panicles per plant (11.00) followed by lines KS-5 and KS-13 respectively. It was observed from pooled data that line KS-22 (17.25) had the highest number of panicles per plant followed by KS-18, KS-7 and KS-20 while KS-1, KS-5 and KS-12 possessed lowest panicles number per plant (11.00) followed by KS-10 and KS-11 respectively. In environment 1, maximum panicle weight was observed in KS-12 (4.23 g) followed by KS-1, KS-8 and KS-9 while minimum panicle weight was observed in KS-4 (2.08 g) followed by KS-10 and KS-2 respectively. In environment 2, panicle weight was found to be maximum (5.23 g) in KS-7 followed by KS-11 KS-1 and KS-4 respectively while KS-21 (2.35) recorded minimum panicle weight followed

**Table 3.** Mean performance of 22 RIL's of rice along with check varieties for 14 characters (pooled over two environments).

| Genotype | Characters | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Plant height (cm) | Days to 50 % flowering | Days to maturity | No. of panicle/plant | Panicle weight (g) | Panicle length (cm) | Florets No./ panicle | No. of grains/ panicle | Fertility % | Grain length (mm) | Grain breadth (mm) | Grain L/B ratio | 1000 grain weight(g) | Grain yield/ plant (g) |
| KS-1 | 121.80 | 114.75 | 145.50 | 11.00 | 4.24 | 23.80 | 219.15 | 164.67 | 75.29 | 8.13 | 3.02 | 2.69 | 21.72 | 27.47 |
| KS-2 | 119.62 | 119.25 | 148.25 | 14.25 | 2.88 | 22.90 | 167.27 | 112.37 | 64.34 | 8.13 | 2.91 | 2.81 | 18.98 | 28.28 |
| KS-3 | 125.75 | 117.00 | 150.25 | 13.25 | 3.05 | 26.64 | 197.95 | 142.05 | 65.68 | 8.49 | 2.83 | 3.02 | 19.78 | 27.96 |
| KS-4 | 116.72 | 114.00 | 147.75 | 14.00 | 3.38 | 24.04 | 197.95 | 140.47 | 69.33 | 8.12 | 2.79 | 2.91 | 16.76 | 27.61 |
| KS-5 | 118.92 | 119.50 | 148.25 | 11.00 | 2.97 | 22.86 | 158.90 | 103.90 | 66.40 | 8.25 | 3.02 | 2.72 | 24.62 | 22.87 |
| KS-6 | 114.87 | 115.75 | 150.50 | 15.50 | 3.12 | 21.99 | 186.65 | 117.60 | 66.87 | 7.95 | 2.50 | 3.21 | 17.37 | 24.08 |
| KS-7 | 119.40 | 114.75 | 149.25 | 16.00 | 3.96 | 25.15 | 192.25 | 123.40 | 63.99 | 8.04 | 3.14 | 2.57 | 24.53 | 33.09 |
| KS-8 | 113.60 | 120.50 | 150.00 | 13.00 | 3.70 | 23.47 | 192.30 | 133.65 | 69.63 | 8.13 | 3.05 | 2.67 | 21.42 | 24.80 |
| KS-9 | 118.22 | 117.50 | 147.00 | 13.00 | 3.74 | 23.64 | 180.60 | 135.02 | 75.93 | 8.03 | 2.94 | 2.68 | 23.09 | 26.67 |
| KS-10 | 124.35 | 125.75 | 152.25 | 12.00 | 2.70 | 23.93 | 149.32 | 105.67 | 68.72 | 9.64 | 2.61 | 3.71 | 24.59 | 27.40 |
| KS-11 | 130.25 | 125.50 | 152.75 | 12.25 | 4.00 | 24.35 | 159.00 | 108.22 | 69.85 | 9.78 | 2.54 | 3.90 | 28.38 | 25.61 |
| KS-12 | 123.40 | 126.50 | 151.25 | 11.00 | 3.69 | 24.03 | 217.57 | 128.62 | 59.76 | 8.74 | 2.20 | 3.19 | 27.29 | 26.78 |
| KS-13 | 135.65 | 123.25 | 152.75 | 13.25 | 3.42 | 21.12 | 190.00 | 143.95 | 75.48 | 7.96 | 2.58 | 3.13 | 20.11 | 32.41 |
| KS-14 | 120.20 | 120.00 | 148.75 | 13.00 | 3.03 | 23.51 | 183.77 | 130.25 | 70.66 | 8.06 | 2.69 | 3.02 | 22.44 | 25.45 |
| KS-15 | 124.45 | 117.50 | 151.15 | 14.25 | 3.15 | 23.06 | 172.60 | 120.40 | 70.72 | 8.09 | 2.91 | 2.78 | 22.17 | 27.69 |
| KS-16 | 127.55 | 116.75 | 146.50 | 13.25 | 2.52 | 24.71 | 182.62 | 135.92 | 74.75 | 7.93 | 2.80 | 2.28 | 21.89 | 27.00 |
| KS-17 | 128.17 | 115.15 | 147.50 | 12.50 | 2.49 | 24.02 | 164.25 | 116.75 | 71.94 | 8.06 | 2.34 | 3.44 | 19.75 | 22.58 |
| KS-18 | 126.65 | 117.00 | 147.00 | 16.00 | 3.41 | 24.46 | 192.70 | 132.92 | 69.66 | 8.09 | 2.73 | 2.95 | 19.12 | 24.42 |
| KS-19 | 126.67 | 117.00 | 145.25 | 13.00 | 2.89 | 24.53 | 181.97 | 124.60 | 69.55 | 8.28 | 2.57 | 3.25 | 20.47 | 28.59 |
| KS-20 | 136.45 | 119.00 | 152.00 | 15.75 | 2.88 | 22.31 | 138.70 | 83.50 | 56.81 | 9.91 | 2.68 | 3.72 | 30.13 | 21.87 |
| KS-21 | 125.27 | 123.25 | 151.25 | 12.75 | 2.49 | 23.54 | 154.52 | 114.42 | 72.11 | 8.25 | 2.48 | 3.33 | 20.38 | 25.19 |
| KS-22 | 139.77 | 122.50 | 153.25 | 17.25 | 3.21 | 23.40 | 164.50 | 103.20 | 58.79 | 10.09 | 2.72 | 3.71 | 26.24 | 25.15 |
| Ranjit* | 108.10 | 117.25 | 147.00 | 12.50 | 2.98 | 23.26 | 235.85 | 136.20 | 55.06 | 8.02 | 2.40 | 3.35 | 16.88 | 26.71 |
| MTU 7029* | 112.37 | 117.25 | 150.75 | 14.25 | 2.57 | 23.24 | 177.20 | 123.52 | 66.16 | 7.79 | 2.64 | 2.94 | 19.14 | 24.84 |
| Mean | 123.26 | 119.19 | 149.43 | 13.55 | 3.19 | 23.66 | 181.56 | 124.22 | 67.81 | 8.42 | 2.73 | 3.10 | 21.97 | 27.33 |
| CD (0.05) | 2.09 | 2.61 | 2.45 | 2.67 | - | 1.11 | 45.84 | 28.06 | 15.17 | - | - | 0.30 | 1.92 | 3.33 |

*Check variety.

by KS-16, KS-17 and MTU-7029 respectively. Pooled data revealed that KS-1 (4.42 g) had maximum panicle weight followed by KS-11 and KS-7 while KS-17 and KS-21 recorded (2.49 g) minimum panicle weight followed by KS-16 and variety MTU-7029 respectively.

In environment 1, maximum panicle length was observed in line KS-3 (25.08 cm) followed by KS-7, KS-19 and KS-16 respectively while KS-13 (19.36cm) recorded minimum panicle length followed by KS-6 and KS-20. In case of environment 2, maximum panicle length was observed in line KS-3 (25.20 cm) followed by KS-11, KS-4 and KS-7 while minimum panicle length was recorded in line KS-13 (22.88 cm) followed by KS-15, KS-6 and KS-5, respectively. From the Pooled data, it was observed that KS-3 (26.64) had maximum panicle length followed by KS-7 and KS-16 while minimum panicle length was recorded in line KS-13 (21.12 cm) followed by KS-6 and KS-20 respectively. Florets number per

panicle was found to be highest in KS-3 (185.05) followed by KS-1 and KS-12 while minimum number of florets per panicle was observed in KS-11 (114.45) followed by KS-20 and KS-10 respectively in environment 1.

In case of environment 2, check variety Ranjit recorded highest number of florets per panicle (296.40) followed by KS-1, KS-12 and KS-13 respectively while minimum florets number per panicle was observed in KS-21 (131.70) followed by KS-20, KS-10 and KS-17 respectively. Variety Ranjit performed best against florets numbers per panicle (235.85), as revealed from pooled data over environments followed by KS-1 and KS-12 while KS-20 recorded minimum florets number per panicle (138.70) followed by lines KS-10 and KS-21, respectively. In environment 1, maximum number of grains per panicle was recorded by KS-9 (139.55) followed by KS-1 and KS-21 while minimum number of grains per panicle was observed in KS-11 (87.85) followed by KS-21 and KS-19.

Similarly, in environment 2, highest numbers of grains per panicle was found in KS-1 (191.25) followed by KS-13, KS-4 and check variety Ranjit, respectively while poor number of grains per panicle was observed in KS-20 (73.80), KS-21 and KS-5. According to pooled data over the two environments maximum number of grains per panicle was observed in KS-1 (164.67) followed by KS-13 and KS-3 while minimum number of grains per panicle was observed in KS-20 (83.50) followed by Ks-22 and KS-5 respectively. Maximum fertility percentage was registered by KS-9 (85.95) followed by KS-15 and KS-1 while minimum fertility percentage was observed in KS-12 (61.10) followed by check variety Ranjit and KS-4 in environment 1. Similarly, in environment 2, maximum fertility percentage was found to be in KS-16 (77.05) followed by KS-1, KS-13 and KS-4 respectively while minimum fertility percentage was occurred in KS-20 (40.04) followed by check variety Ranjit and KS-22. Considering pooled data over two environments it was observed thatKS-9 (75.93) had the highest fertility percentage followed by KS-13 and KS-1 while lowest fertility percentage was observed in check variety Ranjit (55.06) followed by KS-20 and KS-22 respectively.

In environment 1, maximum grain length was observed in KS-22 (10.06 mm) followed by KS-20 and KS-11 while minimum grain length was observed in KS-13 (7.78 mm) followed by variety MTU-7029 and line KS-16 respectively. Similarly, in environment 2, highest grain length was recorded in KS-22 (10.13 mm) followed by KS-20, KS-11 and KS-10 while lowest grain length was registered by variety MTU-7029 (7.72mm) followed by lines KS-8 and KS-6 respectively. Pooled data showed that KS-22 (10.09 mm) had maximum grain length followed by lines KS-20 and KS-11 while minimum grain length was recorded by variety MTU-7029 (7.79 mm)followed by KS-16 and KS-6 respectively. Line KS-7 (3.21 mm) recorded maximum grain breadth in environment 1 followed by KS-8 and KS-5 while variety Ranjit (2.16 mm) showed minimum grain breadth followed by KS-17, KS-13 and KS-11 respectively.

Similarly, in environment 2, KS-7 (3.07 mm) had highest grain breadth followed by lines KS-11, KS-8 and KS-15 while KS-21 (2.43 mm), KS-17 and KS-3 was in the low ranking group in this regards. In pooled data over two environments it was observed that KS-7 had maximum grain breadth (3.14 mm) followed by KS-8 and KS 1 as per pooled data, minimum grain breadth was recorded in KS-l2 (2.20 mm) followed by KS-17 and Ranjit, respectively. In environment 1, line KS-11 (4.38) registered highest grain L/B ratio followed by KS-20 and KS-10 while KS-9 (2.68) had lowest grain L/B ratio followed by KS-8 and KS-2. Similarly in environment 2, KS-22 (3.53) recorded highest grain L/B ratio followed by KS-11, KS-21 and KS-20 respectively while KS-7 (2.59) recorded lowest grain L/B ratio followed by KS-8, KS-5 and KS-1 respectively. In case of pooled data, KS-11 (3.90) recorded highest grain L/B ratio followed by KS-20 and KS-22 while KS-16 (2.28) recorded lowest grain L/B ratio followed by KS-7 and KS-8.1000 grain weight was highest in line KS-20 (31.45 g) followed by KS-11 and KS-5 while lowest 1000 grain weight was found in KS-3 (14.40 g) followed by check variety Ranjit andKS-6 in environment 1.

Similarly, in environment 2, maximum 1000 grain weight was observed in KS-11 (29.90 g ) followed by KS-22, KS-12 and KS-20 while minimum 1000 grain weight was observed in Ranjit (18.97 g) followed by lines KS-4, KS-6 and MTU-7029 respectively. In this regards pooled data revealed that thaKS-20 recorded highest 1000 grain weight (30.13 g) followed by KS-11 and KS-22 while lowest 1000 grain weight was observed in KS-4 (16.76 g) followed by check variety Ranjit and KS-6.In environment 1, grain yield per plant was found to be highest in KS-7 (30.28g) followed by KS-13 and KS-21 while lowest grain yield per plant was observed in KS-18 (15.13 g) followed by KS-22 and KS-6, respectively. Similarly, in environment 2, highest grain yield per plant was recorded in KS-19 (37.11g) followed by KS-4, KS-7 and KS-3 respectively while lowest grain yield per plant was observed in line KS-21 (22.29 g) followed by KS-20 and KS-14, respectively. In pooled data, maximum grain yield per plant was observed in KS-7 (33.09) followed by KS-13 and KS-19 while minimum grain yield per plant was observed in KS-20 (21.87) followed by KS-17, KS-5 and KS-6 respectively.

The range, mean values, mean square values, standard error of difference of the treatment means, critical difference, phenotypic, genotypic and environmental variances, coefficient of variation (CV), genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV), heritability(BS), genetic advance (GA) and genetic advance as percentage of mean of 22 RIL's along with two local checks of rice grown during kharif-2009 (environment 1) and kharif-2010 (environment 2) along with pooled data are presented in Tables 4, 5 and

**Table 4.** Variability and genetic parameters for different quantitative characters in rice duringKharif 2009 [Environment-1].

| Characters | Mean | Range | Mean square | S.E. (diff.) | Variance | | | CV (%) | GCV (%) | PCV (%) | $h^2$ (BS) | G.A. | G.A. as % of mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Phenotypic | Genotypic | Environmental | | | | | | |
| Plant height (cm) | 112.76 | 101.65-135.70 | 146.38** | 1.13 | 73.84 | 72.54 | 1.29 | 1.00 | 7.55 | 7.62 | 0.982 | 17.38 | 15.42 |
| Days to 50 % flowering | 116.35 | 111.00-123.00 | 25.76** | 1.30 | 13.72 | 12.03 | 1.69 | 1.11 | 2.98 | 3.18 | 0.876 | 6.69 | 5.75 |
| Days to maturity | 147.56 | 142.00-152.00 | 20.88** | 1.10 | 11.05 | 9.83 | 1.21 | 0.74 | 2.12 | 2.25 | 0.889 | 6.09 | 4.12 |
| No. of panicle/plant | 12.10 | 10.50-14.50 | 3.21** | 1.35 | 2.52 | 0.69 | 1.82 | 11.16 | 6.89 | 13.11 | 0.276 | 0.90 | 7.45 |
| Panicle weight (g) | 2.81 | 2.08-4.23 | 0.51 | 0.24 | 0.28 | 0.22 | 0.05 | 8.52 | 16.90 | 18.93 | 0.797 | 0.87 | 31.09 |
| Panicle length (cm) | 22.05 | 19.36-25.08 | 2.99** | 0.67 | 1.72 | 1.27 | 0.45 | 3.06 | 5.10 | 5.95 | 0.735 | 1.99 | 9.02 |
| Floret No./panicle | 155.11 | 114.45-183.05 | 682.15** | 20.09 | 543.03 | 139.12 | 403.90 | 12.95 | 7.60 | 15.02 | 0.256 | 12.29 | 7.92 |
| No. of grains/panicle | 110.25 | 87.85-139.55 | 329.38** | 11.68 | 232.92 | 96.46 | 136.45 | 10.59 | 8.90 | 13.84 | 0.414 | 13.01 | 11.80 |
| Fertility % | 71.56 | 61.10-85.95 | 60.77** | 5.64 | 46.29 | 14.48 | 31.81 | 7.88 | 5.31 | 9.50 | 0.312 | 4.38 | 6.12 |
| Grain length (mm) | 8.48 | 7.78-10.06 | 0.92 | 0.10 | 0.46 | 0.43 | 0.01 | 1.21 | 7.95 | 8.04 | 0.977 | 1.37 | 16.19 |
| Grain breadth (mm) | 2.63 | 2.16-3.21 | 0.22 | 0.08 | 0.11 | 0.10 | 0.01 | 3.05 | 12.52 | 12.89 | 0.944 | 0.66 | 25.07 |
| Grain L/B ratio | 3.25 | 2.56-4.38 | 0.53 | 0.10 | 0.27 | 0.26 | 0.01 | 3.28 | 15.67 | 16.01 | 0.957 | 1.02 | 31.60 |
| 1000 grains weight (g) | 20.95 | 14.40-31.45 | 31.62** | 0.58 | 15.98 | 15.64 | 0.34 | 2.79 | 18.87 | 19.08 | 0.978 | 8.05 | 38.46 |
| Grain yield/plant (g) | 21.04 | 15.13-30.28 | 32.76** | 1.63 | 17.71 | 15.04 | 2.66 | 7.76 | 18.43 | 19.99 | 0.849 | 7.36 | 34.9 |

*Significant at 5 % level; ** Significant at 1 % level. CV, Coefficient of variation; GCV, genotypic coefficient of variation; PCV, phenotypic coefficient of variation; GA, genetic advance.

6, respectively. A wide spectrum of variability was noticed for all the characters that offer a good scope of selection for evolving promising lines. The analysis of variance revealed the significant differences among the 24 genotypes against all the characters except panicle weight, grain length, grain breadth and grain L/B ratio.

The wide range of variation that was observed for 10 characters viz. plant height, days to 50 percent flowering, days to maturity, panicles number per plant, panicle length, florets number per panicle, number of grains per panicle, fertility percentage, 1000 grain weight, grain yield per plant, may give good scope for selection on the basis of phenotypic value of component characters. The estimate of phenotypic and genotypic variances were high for florets number per panicle, number of grains per panicles, plant height, and fertility percentage while grain breadth, grain L/B ratio, grain length, panicle length, panicle weight and panicle number per plant showed very low genotypic and phenotypic variances.

In this regard high genotypic and phenotypic variance was reported for number of grains per panicle by Roy et al. (2001). Character like florets number per panicle, number of grains per panicles, plant height and fertility percentage showed high environmental variances simultaneously with high genotypic variances. So, selection for this character would be effective for improvement of these characters in rice.

The magnitude of PCV was higher than GCV for all the characters suggesting the influences of the environmental forces on the expression of these characters. This observation was similar with earlier findings of Chand et al. (2004) and Chaudhary and Singh (1994). High PCV and GCV values were observed in grain yield per plant, 1000 grain weight, grain L/B ratio, grain breadth and panicle weight while number of grains per panicles, grain length, florets number per panicle and plant height exhibited moderate GCV and PCV values therefore there was a large scope for improvements of this characters. In this regard high GCV and PCV for grain yield per plant; 1000 grain weight was earlier reported by Chaubey and Richharia (1993), Nayak and Reddy (2005) and Sarkar et al. (2005). The heritability values varied from 25.6% for florets number per panicle to 98.2% for plant height and genetic advance varied from 4.12% for days to 50% flowering to 38.46 for 1000 grain weight. The heritability estimates were high for plant height, days to maturity, grain length, grain breadth, L/B ratio, 1000 grain weight, panicle weight, panicle length and yield per plant while other characters like fertility percentage and

**Table 5.** Variability and genetic parameters for different quantitative characters in rice during Kharif 2010 [Environment-2].

| Characters | Mean | Range | Mean square | S.E. (diff.) | Variance | | | C V ( % ) | G C V ( % ) | P C V ( % ) | $h^2$ (BS) | G.A. | G.A. as % of mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Phenotypic | Genotypic | Environmental | | | | | | |
| Plant height (cm) | 133.76 | 112.70-145.00 | 170.27** | 0.88 | 85.52 | 84.74 | 0.78 | 0.66 | 6.88 | 6.91 | 0.990 | 18.87 | 14.11 |
| Days to 50 % flowering | 122.04 | 117.00-130.00 | 31.64** | 1.22 | 16.57 | 15.07 | 1.50 | 1.01 | 3.18 | 3.33 | 0.909 | 7.62 | 6.24 |
| Days to maturity | 151.31 | 147.50-155.00 | 7.55** | 1.27 | 4.58 | 2.97 | 1.61 | 0.83 | 1.13 | 1.41 | 0.648 | 2.85 | 1.88 |
| No. of panicle/plant | 15.00 | 11.00-23.00 | 20.00** | 1.23 | 10.76 | 9.23 | 1.52 | 8.22 | 20.26 | 21.86 | 0.858 | 5.80 | 38.67 |
| Panicle weight (g) | 3.85 | 2.35-5.23 | 1.45 | 0.12 | 0.73 | 0.72 | 0.01 | 3.55 | 23.72 | 23.98 | 0.978 | 1.73 | 48.32 |
| Panicle length (cm) | 25.27 | 22.88-28.28 | 3.27** | 0.39 | 1.71 | 1.55 | 0.15 | 1.57 | 4.93 | 5.18 | 0.907 | 2.44 | 9.69 |
| Floret No./panicle | 208.02 | 131.70-257.15 | 2557.35** | 24.22 | 1572.00 | 985.35 | 586.64 | 11.64 | 15.08 | 19.05 | 0.626 | 51.19 | 24.61 |
| No. of grains/panicle | 138.19 | 73.80-191.25 | 1423.74** | 15.45 | 831.30 | 592.44 | 238.86 | 11.18 | 17.61 | 20.86 | 0.712 | 42.32 | 30.63 |
| Fertility % | 64.06 | 40.04-77.05 | 165.03** | 9.03 | 123.28 | 41.74 | 81.53 | 14.09 | 10.08 | 17.33 | 0.338 | 7.74 | 12.08 |
| Grain length (mm) | 8.36 | 7.72-10.13 | 1.06 | 0.16 | 0.54 | 0.51 | 0.02 | 2.00 | 8.59 | 8.82 | 0.948 | 1.44 | 17.24 |
| Grain breadth (mm) | 2.83 | 2.43-3.07 | 0.05 | 0.16 | 0.04 | 0.01 | 0.02 | 5.76 | 4.47 | 7.29 | 0.375 | 0.15 | 5.64 |
| Grain L/B ratio | 2.95 | 2.60-3.53 | 0.17 | 0.18 | 0.10 | 0.06 | 0.03 | 6.28 | 8.85 | 10.86 | 0.665 | 0.44 | 14.88 |
| 1000 grains weight (g) | 22.99 | 18.97-29.90 | 23.83** | 1.27 | 12.73 | 11.10 | 1.62 | 5.54 | 14.48 | 15.51 | 0.872 | 6.41 | 27.87 |
| Grain yield/plant (g) | 33.63 | 22.29-52.24 | 95.97** | 2.07 | 50.13 | 45.83 | 4.30 | 6.16 | 20.13 | 21.05 | 0.914 | 13.33 | 39.65 |

*Significant at 5% level; **Significant at 1% level. CV, Coefficient of variation; GCV, genotypic coefficient of variation; PCV, phenotypic coefficient of variation; GA, genetic advance.

**Table 6.** Variability and genetic parameters for different quantitative characters in rice [pooled over two environments].

| Characters | Mean | Range | Mean square | S.E.(diff.) | Variance | | | CV( % ) | GCV( % ) | PCV( % ) | $h^2$(BS) | G.A. | G.A. as % of mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Phenotypic | Genotypic | Environmental | | | | | | |
| Plant height (cm) | 123.26 | 108.10-139.77 | 158.32** | 1.01 | 79.68 | 78.64 | 1.03 | 0.83 | 7.21 | 7.26 | 0.986 | 18.13 | 14.76 |
| Days to 50 % flowering | 119.19 | 114.00-126.50 | 28.70** | 1.26 | 15.15 | 13.55 | 1.60 | 1.06 | 3.08 | 3.26 | 0.892 | 7.15 | 5.99 |
| Days to maturity | 149.43 | 145.25-153.50 | 14.21** | 1.18 | 7.81 | 6.40 | 1.415 | 0.79 | 1.63 | 1.83 | 0.769 | 4.47 | 3.00 |
| No. of panicle/plant | 13.55 | 11.00-17.25 | 11.60** | 1.29 | 6.64 | 4.96 | 1.673 | 9.69 | 13.57 | 17.49 | 0.567 | 3.35 | 23.06 |
| Panicle weight (g) | 3.19 | 2.49-4.42 | 0.98 | 0.18 | 0.51 | 0.47 | 0.03 | 6.04 | 20.31 | 21.46 | 0.887 | 1.30 | 39.71 |
| Panicle length (cm) | 23.66 | 21.12-26.64 | 3.13** | 0.53 | 1.72 | 1.41 | 0.30 | 2.32 | 5.02 | 5.57 | 0.823 | 2.21 | 9.35 |
| Floret No./panicle | 181.56 | 138.70-235.85 | 1619.78** | 22.15 | 1057.51 | 562.24 | 459.27 | 12.30 | 11.34 | 15.05 | 0.441 | 31.74 | 16.26 |
| No. of grains/panicle | 124.22 | 83.50-164.67 | 876.56** | 13.56 | 532.11 | 344.45 | 187.65 | 10.88 | 13.26 | 15.72 | 0.563 | 27.67 | 21.22 |
| Fertility % | 67.81 | 55.06-75.93 | 112.90** | 7.35 | 84.79 | 28.11 | 56.67 | 10.98 | 7.70 | 9.79 | 0.325 | 6.06 | 9.10 |
| Grain length (mm) | 8.42 | 7.79-10.09 | 0.99 | 0.13 | 0.50 | 0.48 | 0.01 | 161 | 8.27 | 8.43 | 0.962 | 1.40 | 16.71 |
| Grain breadth (mm) | 2.73 | 2.20-3.14 | 0.14 | 0.12 | 0.07 | 0.06 | 0.01 | 4.40 | 8.49 | 10.09 | 0.659 | 0.41 | 15.35 |
| Grain L/B ratio | 3.10 | 2.57-3.90 | 0.35 | 0.14 | 0.18 | 0.16 | 0.02 | 4.78 | 12.26 | 13.43 | 0.811 | 0.73 | 23.24 |
| 1000 grains weight (g) | 21.97 | 16.76-30.13 | 27.73** | 0.93 | 14.35 | 13.37 | 0.98 | 4.17 | 16.68 | 17.29 | 0.925 | 7.23 | 33.17 |
| Grain yield/plant (g) | 27.33 | 21.87-33.09 | 64.36** | 1.85 | 33.92 | 30.21 | 3.48 | 6.96 | 19.28 | 20.52 | 0.881 | 10.34 | 37.32 |

*Significant at 5% level; **Significant at 1% level. CV, Coefficient of variation; GCV, genotypic coefficient of variation; PCV, phenotypic coefficient of variation; GA, genetic advance.

number of grain per panicle showed moderate heritability and characters like panicle number per plant and florets number per panicle, possessed low heritability. This finding corroborated the earlier findings of Sawant and Patil (1995) and Ashvani et al. (1997). High heritability coupled with moderate to high genetic advance for plant height, panicle weight, grain length, grain breadth, grain L/B ratio, 1000 grain weight and grain yield per plant indicated predominance of additive gene action controlling for this characters and therefore, selection based on phenotypic performance would be effective against this characters.

The character like days to 50% flowering, days to maturity, panicle length, fertility percentage and number of grains per panicle showed moderate to high heritability values associated with moderate to low genetic, advance, suggesting that the inheritance of such traits might be under control of both additive and non-additive gene effects. Low heritability estimates along with low genetic advance observed for panicle number per plant and florets number per panicle suggested that dominances and epistatic gene effects might be operating in the inheritance of these traits. Similarly, in environment 2, the heritability values varied from 38.80% for fertility percentage to 99% for plant height and genetic advance as percentage of mean varied from 1.88% for days to maturity to 39.65% for grain yield per plant.

High heritability coupled with moderate to high genetic advance as percentage of mean for plant height, panicle numbers per plant, panicle weight, grain length, 1000 grain weight and grain yield per plant indicated predominance of additive gene action controlling for this characters and therefore direct selection based on phenotypic performance would be effective against this characters. Low heritability along with low genetic advance observed for grain breadth and fertility percentage suggested that dominance and epistatic gene action were responsible for controlling this traits. In pooled analysis, the heritability values ranged from 32.50% in fertility percentage to 98.60 for plant height and genetic advance as percentage of mean 3% in days to maturity to 37.32% for grain yield per plant. High heritability coupled with moderate to high genetic advance as percent of mean for plant height, panicle weight, grain length, grain L/B ratio, 1000 grain weight and yield per plant indicated predominance of additive gene action for controlling these characters while low heritability estimates along with low genetic advance observed for fertility percentage and florets number per panicle suggested that dominance and epistatic genes were involved in the controlling of this traits.

The genotypic and phenotypic correlation coefficients among the biometrical traits estimated over environment 1 and environment 2 and pooled data are presented in Tables 7, 8 and 9, respectively. In general genotypic correlation coefficients were higher than their corresponding phenotypic correlation coefficients. This finding was supported earlier by Chaudhary and Singh (1994). Grain yield per plant was significantly positively correlated with number of grains per panicle at genotypic level only in environments 1. In environments 2, it had significant positive correlation against panicle weight and number of grains per panicle at genotypic and phenotypic level whereas against floret number per panicle at genotypic level only. In pooled data analysis it had positive significant correlation against floret number per panicle and number of grains per panicle at genotypic level only.

The genotypic associations of yield and its attributing characters in environment 1, environment 2 and pooled data are presented in Table 10, 11 and 12, respectively. Path coefficient analysis was based on correlation coefficient using grain yield as the dependent factor (effect) and fix other quantitative characters viz., plant height, days to 50% flowering, days to maturity, panicle number per plant, panicle weight, panicle length, floret number per panicle, number of grains per panicle, fertility percentage, grain length, grain breadth, grain L/B ratio and 1000 grain weight as independent factor (Causes). Correlation coefficient of each independent quantitative character was partitioned into direct and indirect effects towards grain yield. Half of the characters viz. plant height, days to 50% flowering, days to maturity, panicle weight, panicle length and number of panicles per plant possessed positive direct effect and other half of the characters, that is, panicles number per plant, florets per panicle, fertility percentage, grain length, grain breadth, grain L/B ratio and 1000 grain weight incurred negative direct effect toward grain yield per plant in environment 1. Number of grains per panicle imparted the maximum positive direct effect on grain yield per plant followed by plant height, days to 50% flowering, panicle length, panicle weight and days to maturity respectively. The present findings are supported by Reuben and Kisanga (1989), Padmavathi et al. (1996) and Sarkar et al. (2005).

Similarly, florets number per panicle imparted maximum negative direct effect on grain yield followed by fertility percentage, grain length, grain L/B ratio, panicle number per plant, grain breadth and 1000 grain weight respectively. Number of grains per panicle had high positive direct effect and also had highly positive significant correlation coefficient with grain yield. Therefore, direct selection for number of grains per panicle would be effective for yield improvement in rice. Majority of characters viz. days to 50% flowering, days to maturity, panicle number per plant, panicle weight, panicle length, number of florets per panicle, grain breadth, grain L/B ratio incurred positive direct effect toward grain yield and rest of the characters recorded negative direct effects in this regards in environment 2. A maximum positive direct effect was imparted by grain L/B ratio followed by number of grains per panicle, grain breadth and panicles number per plant. Panicle weight and number of grains per panicle were positively

**Table 7.** Genotypic (G) and phenotypic (P) correlation coefficients for grain yield and its attributing characters during Kharif 2009 [Environment-1].

| Characters | | Days to 50% flowering | Days to maturity | No. of panicle/plant | Panicle weight (g) | Panicle length (cm) | Floret No./ panicle | No. of grains/ Panicle | Fertility % | Grain length (mm) | Grain breadth (mm) | Grain L/B ratio | 1000 grains weight (g) | Grain yield/ plant (g) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plant height (cm) | G | 0.468* | 0.464* | -0.037 | -0.035 | -0.168 | -0.492* | -0.397* | 0.050 | 0.576** | -0.190 | 0.448* | 0.442* | 0.143 |
| | P | 0.433* | 0.438* | -0.059 | -0.032 | -0.158 | -0.274 | -0.274 | 0.039 | 0.557** | -0.189 | 0.434* | 0.429* | 0.139 |
| Days to 50 % flowering | G | | 0.731** | 0.069 | 0.203 | -0.324 | -0.585** | -0.479* | 0.081 | 0.503* | -0.397* | 0.568** | 0.459* | 0.045 |
| | P | | 0.695** | 0.001 | 0.170 | -0.206 | -0.181 | -0.220 | -0.007 | 0.466* | -0.383 | 0.519** | 0.416* | 0.048 |
| Days to maturity | G | | | 0.026 | -0.008 | -0.249 | -0.609** | -0.652** | -0.185 | 0.529** | -0.170 | 0.425* | 0.377 | 0.016 |
| | P | | | 0.026 | 0.045 | -0.201 | -0.252 | -0.350 | -0.098 | 0.506** | -0.150 | 0.379 | 0.360 | 0.039 |
| No. of panicle/plant | G | | | | -0.164 | 0.151 | 0.528** | 0.454* | 0.083 | -0.556** | 0.086 | -0.387 | -0.454* | 0.264 |
| | P | | | | -0.140 | -0.074 | -0.203 | -0.052 | 0.233 | -0.248 | 0.055 | -0.186 | -0.189 | 0.136 |
| Panicle weight (g) | G | | | | | -0.008 | 0.359 | 0.280 | -0.049 | -0.001 | 0.159 | -0.150 | 0.374 | -0.053 |
| | P | | | | | 0.016 | 0.245 | 0.234 | -0.063 | 0.009 | 0.146 | -0.166 | 0.346 | -0.019 |
| Panicle length (cm) | G | | | | | | 0.530** | 0.465* | -0.001 | -0.048 | 0.485* | -0.400* | 0.008 | 0.071 |
| | P | | | | | | 0.513** | 0.333 | -0.329 | -0.040 | 0.407* | -0.334 | -0.009 | 0.131 |
| Floret No./panicle | G | | | | | | | 0.763** | -0.161 | -0.838** | 0.324 | -0.752** | -0.615** | 0.389 |
| | P | | | | | | | 0.766** | -0.465* | -0.459* | 0.217 | -0.432* | -0.317 | 0.216 |
| No. of grains/Panicle | G | | | | | | | | 0.517** | -0.731** | 0.375 | -0.711** | -0.384 | 0.506* |
| | P | | | | | | | | 0.208 | -0.504* | 0.291 | -0.514** | -0.253 | 0.285 |
| Fertility % | G | | | | | | | | | -0.016 | 0.099 | -0.072 | 0.241 | 0.264 |
| | P | | | | | | | | | -0.000 | 0.048 | -0.037 | 0.129 | 0.041 |
| Grain length (mm) | G | | | | | | | | | | -0.101 | 0.634** | 0.641** | -0.384 |
| | P | | | | | | | | | | -0.105 | 0.631** | 0.631** | -0.352 |
| Grain breadth (mm) | G | | | | | | | | | | | -01.829 | 0.077 | 0.213 |
| | P | | | | | | | | | | | -0.824 | 0.079 | 0.178 |
| Grain L/B ratio | G | | | | | | | | | | | | 0.275 | -0.353 |
| | P | | | | | | | | | | | | 0.264 | -0.317 |
| 1000 grains weight (g) | G | | | | | | | | | | | | | 0.026 |
| | P | | | | | | | | | | | | | 0.019 |

*Significant at 5% level; **Significant at 1% level; G, genotypic correlation coefficient; P, phenotypic correlation coefficient.

associated with grain yield and also recorded high and very high positive direct effects towards grain yield respectively. Therefore, direct selection for these two characters would be effective in yield improvement of rice.

Florets number per panicle was positively associated with grain yield but it had high negative direct effects toward grain yield. Therefore, high amount of indirect effects of number of grains per panicle, grain length and grain breadth were responsible for positive significant correlation. Therefore, during selection these characters should be considered for selection along with florets number per plant. Pooled analysis showed that majority of the characters viz. plant height, days to 50% flowering, days to maturity, panicle weight, panicle length, number of grains per panicle, grain breadth and grain L/B ratio had positive direct effects toward grain yield. Number of grains per panicle incurred a high positive direct effect simultaneously with significant positive

**Table 8.** Genotypic (G) and phenotypic (P) correlation coefficients for grain yield and its attributing charactersduringKharif 2010 [Environment-2].

| Characters | | Days to 50 % flowering | Days to maturity | No. of panicle/plant | Panicle weight (g) | Panicle length (cm) | Floret No./ panicle | No. of grains/ Panicle | Fertility % | Grain length (mm) | Grain breadth (mm) | Grain L/B ratio | 1000 grains weight (g) | Grain yield/ plant (g) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plant height (cm) | G | 0.279 | 0.333 | 0.138 | -0.116 | 0.186 | -0.408* | -0.220 | 0.110 | 0.462* | 0.076 | 0.399 | 0.431* | -0.185 |
| | P | 0.267 | 0.270 | 0.137 | -0.105 | 0.169 | -0.314 | -0.188 | 0.066 | 0.448* | 0.054 | 0.317 | 0.389 | -0.171 |
| Days to 50 % flowering | G | | 0.603** | -0.105 | -0.066 | -0.072 | -0.323 | -0.422* | -0.343 | 0.764** | 0.347 | 0.549** | 0.777** | -0.271 |
| | P | | 0.611** | -0.101 | -0.053 | -0.073 | -0.214 | -0.310 | -0.168 | 0.702** | 0.148 | 0.465* | 0.686** | -0.239 |
| Days to maturity | G | | | 0.316 | 0.029 | -0.333 | -0.373 | -0.493* | -0.464* | 0.613** | -0.003 | 0.590** | 0.555** | 0.071 |
| | P | | | 0.247 | 0.044 | -0.253 | -0.200 | -0.235 | -0.152 | 0.477* | 0.065 | 0.331 | 0.446* | 0.077 |
| No. of panicle/plant | G | | | | 0.030 | 0.091 | -0.189 | -0.308 | -0.578** | 0.368 | -0.105 | 0.393 | 0.206 | 0.352 |
| | P | | | | 0.044 | 0.057 | -0.135 | -0.288 | -0.344 | 0.323 | 0.063 | 0.199 | 0.169 | 0.324 |
| Panicle weight (g) | G | | | | | 0.027 | 0.474* | 0.519** | 0.257 | 0.021 | 0.548** | -0.268 | 0.139 | 0.504* |
| | P | | | | | 0.246 | 0.407* | 0.450* | 0.175 | 0.015 | 0.354 | -0.237 | 0.110 | 0.499* |
| Panicle length (cm) | G | | | | | | 0.159 | 0.357 | 0.382 | 0.172 | -0.131 | 0.225 | 0.070 | 0.165 |
| | P | | | | | | 0.122 | 0.223 | 0.100 | 0.163 | -0.030 | 0.136 | 0.060 | 0.127 |
| Floret No./panicle | G | | | | | | | 0.947** | 0.149 | -0.314 | 0.261 | -0.455* | -0.252 | 0.474* |
| | P | | | | | | | 0.708** | -0.030 | -0.267 | 0.235 | -0.399* | -0.275 | 0.392 |
| No. of grains/Panicle | G | | | | | | | | 0.533** | -0.508** | 0.274 | -0.641** | -0.511 | 0.452* |
| | P | | | | | | | | 0.558** | -0.402* | 0.118 | -0.414* | -0.408* | 0.437* |
| Fertility % | G | | | | | | | | | -0.702* | 0.093 | -0.706** | -0.577** | 0.148 |
| | P | | | | | | | | | -0.386 | -0.052 | -0.252 | -0.303 | 0.190 |
| Grain length (mm) | G | | | | | | | | | | 0.155 | 0.865** | 0.893** | -0.179 |
| | P | | | | | | | | | | 0.123 | 0.704** | 0.809** | -0.181 |
| Grain breadth (mm) | G | | | | | | | | | | | -0.365 | 0.630** | 0.053 |
| | P | | | | | | | | | | | -0.614** | 0.391 | 0.054 |
| Grain L/B ratio | G | | | | | | | | | | | | 0.522** | -0.213 |
| | P | | | | | | | | | | | | 0.374 | -0.193 |
| 1000 grains weight (g) | G | | | | | | | | | | | | | -0.356 |
| | P | | | | | | | | | | | | | -0.325 |

*Significant at 5% level; **Significant at 1 % level, G, Genotypic correlation coefficient . P, Phenotypic correlation coefficient.

correlation with grain yield per plant. Florets number per panicle imparted the maximum negative direct effect followed by grain breadth, fertility percentage and panicle numbers per plant. Significant positive correlation simultaneously with highest amount of negative direct effect of florets number per plant suggested that high positive indirect effects of number of grains per panicle, grain length, panicle length etc. were responsible for incurring positive significant yield correlation. Therefore the above mentioned three characters should be considered during selection simultaneous with florets number per plant in rice.

## Conclusion

The performance of 22 RIL's revealed that two

**Table 9.** Genotypic (G) and phenotypic (P) correlation coefficients for grain yield and its attributing characters [pooled over two environments].

| Characters | | Days to 50 % flowering | Days to maturity | No. of panicle/plant | Panicle weight (g) | Panicle length (cm) | Floret no./ panicle | No. of grains/ Panicle | Fertility % | Grain length (mm) | Grain breadth (mm) | Grain L/B ratio | 1000 grains weight (g) | Grain yield/ plant (g) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plant height (cm) | G | 0.373 | 0.398* | 0.050 | -0.075 | 0.177 | -0.450* | -0.308 | 0.080 | 0.519** | -0.057 | 0.423* | 0.436* | -0.021 |
| | P | 0.350 | 0.354 | 0.039 | -0.068 | 0.163 | -0.294 | -0.231 | 0.052 | 0.502** | -0.067 | 0.375 | 0.233 | -0.016 |
| Days to 50 % flowering | G | | 0.667** | -0.018 | 0.068 | -0.198 | -0.454* | -0.450* | -0.131 | 0.633** | -0.025 | 0.558** | 0.618** | -0.113 |
| | P | | 0.653** | -0.050 | 0.058 | -0.139 | -0.197 | -0.265 | -0.087 | 0.584** | -0.117 | 0.492* | 0.551** | -0.095 |
| Days to maturity | G | | | 0.171 | 0.010 | -0.291 | -0.491* | -0.572** | -0.324 | 0.571** | -0.086 | 0.507** | 0.466* | 0.043 |
| | P | | | 0.136 | 0.044 | -0.227 | -0.226 | -0.292 | -0.125 | 0.491* | -0.042 | 0.360 | 0.403* | 0.058 |
| No. of panicle/plant | G | | | | -0.067 | 0.121 | 0.169 | 0.073 | -0.247 | -0.094 | -0.095 | -0.390 | -0.124 | 0.308 |
| | P | | | | -0.048 | -0.065 | -0.169 | -0.170 | -0.055 | 0.037 | 0.059 | 0.192 | -0.010 | 0.230 |
| Panicle weight (g) | G | | | | | 0.131 | 0.416* | 0.399* | 0.104 | 0.010 | 0..353 | -0.209 | 0.256 | 0.225 |
| | P | | | | | 0.131 | 0.326 | 0.342 | 0.057 | 0.012 | 0.250 | -0.201 | 0.228 | 0.240 |
| Panicle length (cm) | G | | | | | | 0.344 | 0.411** | 0.190 | 0.062 | 0.177 | -0.087 | 0.039 | 0.118 |
| | P | | | | | | 0.317 | 0.278 | -0.114 | 0.061 | 0.188 | -0.099 | 0.025 | 0.129 |
| Floret No./panicle | G | | | | | | | 0.855** | -0.155 | -0.576** | 0.292 | -0.603** | -0.433* | 0.431* |
| | P | | | | | | | 0.737** | -0.247 | -0.363 | 0.226 | -0.415* | -0.296 | 0.304 |
| No. of grains/Panicle | G | | | | | | | | 0.525** | -0.613** | 0..324 | -0.676** | -0.447* | 0.479* |
| | P | | | | | | | | 0.383 | -0.453* | 0.204 | -0.464* | -0.330 | 0.361 |
| Fertility % | G | | | | | | | | | -0.359 | 0.096 | -0.389 | -0.168 | 0.206 |
| | P | | | | | | | | | -0.193 | -0.050 | -0.144 | -0.087 | 0.115 |
| Grain length (mm) | G | | | | | | | | | | 0.027 | 0.749** | 0.676** | -0.281 |
| | P | | | | | | | | | | 0.114 | 0.667** | 0.720** | -0.266 |
| Grain breadth (mm) | G | | | | | | | | | | | -0.597** | 0.353 | 0.133 |
| | P | | | | | | | | | | | -0.719** | 0.235 | 0.116 |
| Grain L/B ratio | G | | | | | | | | | | | | 0.398* | -0.283 |
| | P | | | | | | | | | | | | 0.319 | -0.255 |
| 1000 grains weight (g) | G | | | | | | | | | | | | | -0.165 |
| | P | | | | | | | | | | | | | -0.153 |

*Significant at 5% level, **Significant at 1 % level, G: Genotypic correlation coefficient; P: phenotypic correlation.

lines viz; KS-7 and KS-13 were promising in respect of grain yield and some other yield related traits. GCV, PCV, heritability and genetic advance highlighted the importance of plant height, panicle weight, grain length, grain L/B ratio and 1000 grain weight for yield improvement in rice through selection and hybridization. Correlation and path analysis showed that number of grains per panicle and floret number per panicle were the most important yield controlling characters in rice. Therefore, number of grains per panicle and florets number per panicle should be considered during hybridization and selection for yield improvement in rice.

## Conflict of Interests

The author(s) have not declared any conflict of

**Table 10.** Matrix of direct (diagonal) and indirect effect of yield attributing characters on yield of rice during kharif 2009 [Environment-1].

| Characters | Plant height (cm) | Days to 50 % flowering | Days to maturity | No. of panicle/plant | Panicle weight (g) | Panicle length (cm) | Floret no./ panicle | No. of grains/ Panicle | Fertility % | Grain length (mm) | Grain breadth (mm) | Grain L/B ratio | 1000 grains weight (g) | Genotypic yield correlation |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plant height (cm) | **0.757** | 0.309 | 0.010 | 0.016 | -0.001 | -0.111 | 1.546 | -1.059 | -0.084 | -0.887 | 0.055 | -0.360 | -0.049 | 0.143 |
| Days to 50 % flowering | 0.354 | **0.660** | 0.017 | -0.031 | 0.007 | -0.213 | 1.837 | -1.279 | -0.138 | -0.775 | 0.115 | -0.456 | -0.051 | 0.045 |
| Days to maturity | 0.351 | 0.048 | **0.023** | -0.012 | -0.002 | -0.146 | 1.913 | -1.740 | 0.313 | -0.815 | 0.049 | -0.341 | -0.042 | 0.016 |
| No. of panicle/plant | -0.027 | 0.045 | 0.006 | **-0.456** | -0.005 | 0.099 | -1.658 | 1.212 | -0.140 | 0.857 | -0.024 | 0.310 | 0.051 | 0.264 |
| Panicle weight (g) | -0.026 | 0.133 | -0.001 | 0.075 | **0.035** | -0.005 | -1.129 | 0.747 | 0.083 | 0.002 | -0.046 | 0.120 | -0.042 | -0.053 |
| Panicle length (cm) | -0.012 | -0.213 | -0.005 | -0.069 | -0.003 | **0.659** | -1.166 | 1.241 | 0.000 | 0.073 | -0.141 | 0.321 | -0.008 | 0.071 |
| Floret No./panicle | -0.372 | -0.385 | -0.014 | -0.240 | 0.012 | 0.349 | **-3.142** | 2.038 | 0.273 | 1.291 | -0.094 | 0.604 | 0.069 | 0.389 |
| No. of grains/Panicle | -0.300 | -0.316 | -0.015 | -0.207 | 0.009 | 0.306 | -2.398 | **2.671** | -0.876 | 1.127 | -0.109 | 0.571 | 0.043 | 0.506** |
| Fertility % | 0.037 | 0.053 | -0.004 | -0.037 | -0.001 | -0.003 | 0.506 | 1.380 | **-1.696** | 0.023 | -0.028 | 0.058 | -0.027 | 0.264 |
| Grain length (mm) | 0.436 | 0.332 | 0.012 | 0.253 | -0.005 | -0.031 | 2.633 | -1.953 | 0.026 | **-1.541** | 0.029 | -0.509 | -0.072 | -0.384 |
| Grain breadth (mm) | -0.144 | -0.261 | -0.004 | -0.039 | 0.005 | 0.319 | -1.018 | 1.002 | -0.168 | 0.155 | **-0.291** | 0.665 | -0.008 | 0.213 |
| Grain L/B ratio | 0.339 | 0.375 | 0.010 | 0.176 | -0.005 | -0.264 | 2.362 | -1.899 | -0.122 | -0.977 | 0.241 | **-0.803** | -0.030 | -0.353 |
| 1000 grains weight (g) | 0.334 | 0.302 | 0.008 | 0.207 | 0.013 | 0.004 | 1.933 | -1.025 | -0.409 | -0.988 | -0.022 | -0.221 | **-0.112** | 0.026 |

*Significant at 5% level, **Significant at 1% level, residual effect = 0.4653.

**Table 11.** Matrix of direct (diagonal) and indirect effect of yield attributing characters on yield of rice during kharif 2010 [Environment-2].

| Characters | Plant height (cm) | Days to 50 % flowering | Days to maturity | No. of panicle/plant | Panicle weight (g) | Panicle length (cm) | Floret No./ panicle | No. of grains/ Panicle | Fertility % | Grain length (mm) | Grain breadth (mm) | Grain L/B ratio | 1000 grains weight (g) | Genotypic yield correlation |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plant height (cm) | **-0.194** | 0.051 | 0.129 | 0.062 | -0.027 | 0.014 | 0.398 | -0.344 | -0.048 | -0.815 | 0.077 | 0.710 | -0.199 | -0.185 |
| Days to 50 % flowering | -0.054 | **0.158** | 0.235 | -0.047 | -0.015 | -0.005 | 0.314 | -0.658 | 0.152 | -1.346 | 0.351 | 0.977 | -0.359 | -0.271 |
| Days to maturity | -0.064 | 0.112 | **0.389** | 0.142 | 0.006 | -0.025 | 0.363 | -0.769 | 0.205 | -1.081 | -0.002 | 1.050 | -0.266 | 0.071 |
| No. of panicle/plant | -0.026 | -0.019 | 0.123 | **0.451** | 0.007 | 0.006 | 0.184 | -0.480 | 0.256 | -0.649 | -0.106 | 0.700 | -0.095 | 0.352 |
| Panicle weight (g) | 0.022 | -0.012 | 0.011 | 0.013 | **0.237** | 0.020 | -0.462 | 0.810 | -0.114 | -0.036 | 0.555 | -0.477 | -0.064 | 0.504* |
| Panicle length (cm) | -0.036 | -0.013 | -0.129 | 0.041 | 0.064 | **0.075** | -0.155 | 0.557 | -0.169 | -0.304 | -0.132 | 0.400 | -0.032 | 0.165 |
| Floret No./panicle | 0.076 | -0.059 | -0.145 | -0.085 | 0.112 | 0.012 | **-0.975** | 1.478 | -0.006 | 0.544 | 0.264 | -0.811 | 0.116 | 0.474* |
| No. of grains/Panicle | 0.042 | -0.078 | -0.192 | -0.138 | 0.0123 | 0.026 | -0.924 | **1.560** | -0.236 | 0.896 | 0.277 | -1.142 | 0.236 | 0.452* |
| Fertility % | -0.021 | -0.063 | -0.180 | -0.260 | 0.061 | 0.028 | -0.145 | 0.832 | **-0.444** | 1.237 | 0.094 | -1.257 | 0.266 | 0.148 |
| Grain length (mm) | -0.089 | 0.141 | 0.239 | 0.166 | 0.004 | 0.013 | 0.306 | -0.793 | 0.311 | **-1.763** | 0.156 | 1.540 | -0.412 | -0.179 |
| Grain breadth (mm) | -0.014 | 0.064 | -0.001 | -0.047 | 0.130 | -0.009 | -0.254 | 0.427 | -0.041 | -0.272 | **0.012** | -0.649 | -0.291 | 0.053 |
| Grain L/B ratio | -0.077 | 0.101 | 0.229 | 0.177 | -0.063 | 0.016 | 0.443 | -1.000 | 0.313 | -1.525 | -0.369 | **1.781** | -0.241 | -0.213 |
| 1000 grains weight (g) | -0.083 | 0.144 | 0.216 | 0.092 | 0.032 | 0.005 | 0.245 | -0.797 | 0.256 | -1.574 | 0.638 | 0.930 | **-0.462** | -0.356 |

*Significant at 5% level, ** Significant at 1 % level, residual effect = 0.4047.

**Table 12.** Matrix of direct (diagonal) and indirect effect of yield attributing characters on yield of rice [pooled over two environments].

| Characters | Plant height (cm) | Days to 50 % flowering | Days to maturity | No. of panicle/plant | Panicle weight (g) | Panicle length (cm) | Floret No./ panicle | No. of grains/ Panicle | Fertility % | Grain length (mm) | Grain breadth (mm) | Grain L/B ratio | 1000 grains weight (g) | Genotypic yield correlation |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plant height (cm) | **0.281** | 0.180 | 0.070 | 0.039 | -0.014 | -0.048 | 0.972 | -0.701 | -0.066 | -0.849 | 0.066 | 0.175 | -0.214 | -0.021 |
| Days to 50 % flowering | 0.150 | **0.422** | 0.126 | -0.039 | -0.011 | -0.109 | 1.076 | -0.969 | 0.141 | -1.069 | 0.233 | 0.260 | -0.205 | -0.113 |
| Days to maturity | 0.143 | 0.297 | **0.206** | 0.065 | 0.003 | -0.094 | 1.138 | -1.255 | 0.259 | -0.948 | 0.023 | 0.354 | -0.149 | 0.043 |
| No. of panicle/plant | -0.027 | 0.013 | 0.061 | **-0.453** | 0.006 | 0.053 | -0.736 | 0.365 | 0.058 | 0.103 | -0.065 | 0.505 | -0.022 | 0.308 |
| Panicle weight (g) | -0.024 | 0.060 | 0.056 | 0.044 | **0.136** | 0.012 | -0.796 | 0.778 | -0.015 | -0.041 | 0.254 | -0.178 | 0.053 | 0.225 |
| Panicle length (cm) | -0.081 | -0.113 | 0.067 | -0.014 | 0.031 | **0.367** | -0.19 | 0.899 | -0.084 | -0.222 | -0.137 | 0.360 | -0.016 | 0.118 |
| Floret No./ panicle | -0.146 | -0.222 | -0.079 | -0.163 | 0.062 | 0.180 | **-2.095** | 1.758 | 0.103 | 0.229 | 0.084 | -0.103 | 0.092 | 0.431* |
| No. of grains/ Panicle | -0.128 | -0.197 | -0.103 | -0.173 | 0.066 | 0.166 | -1.661 | **2.116** | -0.556 | 0.393 | 0.084 | -0.285 | 0.139 | 0.479* |
| Fertility % | -0.029 | -0.058 | -0.092 | -0.149 | 0.029 | 0.014 | 0.180 | 1.106 | **-1.070** | 0.630 | 0.032 | -0.599 | 0.119 | 0.206 |
| Grain length (mm) | 0.173 | 0.236 | 0.125 | 0.210 | 0002 | -0.022 | 1.470 | -1.373 | 0.168 | **-1.652** | 0.093 | 0.515 | -0.242 | -0.281 |
| Grain breadth (mm) | -0.079 | -0.098 | -0.002 | -0.043 | 0.068 | 0.155 | -0.636 | 0.715 | -0.104 | -0.058 | **0.360** | .657 | -0.149 | 0.133 |
| Grain L/B ratio | 0.131 | 0.238 | 0.119 | 0.176 | -0.034 | -0.123 | 1.430 | -1.450 | 0.218 | -1.231 | -0.063 | **0.488** | -0.136 | -0.283 |
| 1000 grains weight (g) | 0.125 | 0.223 | 0.112 | 0.150 | 0.023 | 0.005 | 1.089 | -0.911 | -0.076 | -1.281 | 0.307 | 0.354 | **-0.287** | -0.165 |

*Significant at 5% level; **Significant at 1% level; residual effect = 0.4350.

interests.

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*Full Length Research Paper*

# ‘Do-sono’ passion fruit: Ecogeographical prospecting and phenotypic dispersion in transition areas between the *caatinga* and *cerrado* of Brazil

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**This study performed eco-geographical prospecting on five populations of wild ‘de-sono’ passion fruit plants in the rural zones of five towns from two distinct agroecological units in the state of Bahia. Ripe fruit from plants from these populations that had fallen to the ground were physicochemically characterized and had their phenotypic variability dispersion among the populations estimated via uni- and multivariate variance and grouping. Among them, the five populations presented univariate and multivariate differentiation. The large majority of multivariate contrasts between the location pairs were shown to be statistically significant Hotelling's T-squared test. The five populations were arranged in a dendogram containing two groups, since a standard of spatial variation between the populations has still not been detected by Mantel Test, although it presents coherence in regards to the agroecological units from which they are allocated. The results are discussed in terms of genetic variability among populations, and the relationships between this diversity, the environmental factors of the ecogeographical units where the plants of the sampled populations were collected, and the implications of the results in terms of selection and use for the genetic improvement of passiflora.**

**Key words:** ‘Do-sono’ passion fruit, native fruits, genetic variability, selection, genetic improvement.

## INTRODUCTION

The Passifloraceae family comprises approximately 600 species, about 150 of which are native to Brazil, making our country the principal center of dispersion, and consequently diversity, for this family of species (Oliveira and Ruggiero, 2005; Nascimento, 2006). The cultivation of passiflora stands out in Brazil, considered to be the leading producer of passion fruit (Santos, 2006). Bahia produces about 77 thousand tons of fruit on an area of 7.8 thousand hectares of passiflora, making the state the biggest national producer (Roncatto et al., 2005).

According to Ferreira (1998), more than 50 species of passiflora are primarily cultivated, due to the nutritional

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**Table 1.** Classification of large geoenvironmental units from the towns in which samples of wild populations of 'do-sono' passion fruit (*P. setacea)* were collected.

| City | Large Landscape" Unit Chapada Diamantina" | | Large Landscape Unit "Superfícies Retrabalhadas | | Geoenvironmental Unit in which the collections were taken/samples were collected |
|---|---|---|---|---|---|
| | UG C5[1] | UG C7[2] | UG E2[3] | UG E7 | |
| Vitoria da Conquista/VC | Yes | No | Yes | Yes | C5 |
| Piripá/PP | Yes | Yes | No | No | C7 |
| Mortugaba/MO | No | Yes | No | No | C7 |
| Cordeiros/CO | No | Yes | No | No | C7 |
| Condeúba/CD | No | Yes | No | No | C7 |

Note: [1], [2] and [3] = C5, C7 and E2 = 'Planalto de Vitória da Conquista' and 'Piemontes dos Altos Maciços Centrais and Relevos Associados' 'Maciços and Serras Altas', respectively. [1] The meaning of the abbreviations of the populations is found in Table 2; [2] General average; [3] variation coefficient, in percentage.

qualities of their fruits and pharmaceutical properties of their juices, skins and seeds. Among these is highlighted the 'do-sono' passion fruit (*Passiflora setacea* DC)*,* a plant native to the *cerrado* and *caatinga* biomes in Brazil (Oliveira e Ruggiero; 2005). Its unique fruit, smell and flavors are highly valued in the regions where it occurs, and it is sold in open-air markets of some cities in the Brazilian Northeast (Oliveira, Personal communication, 2010) and the Central-West.

Because they are not commercially grown, fruits of this species are collected from different populations of naturally occurring plants thus explaining the enormous diversity of the physicochemical characteristics of the fruits found for sale in these markets. Species of native plants that are sexually reproduced and have not yet been domesticated, such as the 'do-sono' passion fruit variety, exhibit a lot of heterogeneity in regard to their fruits, both in terms of their physical and chemical characteristics (Cardoso-Silva et al., 2007).

A good survey of functional biodiversity in the scope of vegetable genetic resources, as is the case of characteristics related to the shape and flavor of fruits, requires that the sampling is done to reach the greatest quantity of useful genetic variation in the lowest number of samples possible, keeping in mind the type of phenotypic variability desired. Conceived by Maxted et al. (1995), the methodology of 'ecogeographical prospecting' responds to these issues by taking geographical and ecological aspects into consideration for the purpose of definition of sampling points.

Presently, only one study of the morpho-agronomic dispersion of species of the *Passiflora* spp. genus, based on the principal of 'ecogeographical prospecting' has been done by Araújo (2007), who studied the distribution of the 'do-mato' passion fruit (*Passiflora cincinnata* Mast) in ecogeographical areas of the Brazilian northeast.

This study involves the ecogeographical prospect and study of the dispersion of physicochemical phenotypic variability of fruits via uni- and multivariate variance and grouping analyses of five natural populations of 'de-sono' passion fruit, available in the southwest of Bahia pertaining to geoenvironmental units of the northeast, in order to guide future collection studies focused on the preservation and pre-improvement of the species.

## MATERIALS AND METHODS

### Ecogeographical collection of the fruits

The fruits were collected from plants from five populations, located in the Bahian municipalities of Vitória da Conquista, Cordeiros, Piripá, Condeúba and Mortugaba, pertaining to two ecogeographical areas in the state of Bahia (Table 1), according to terminology in the Northeast Agroecological Zoning (ZANE) (Silva et al., 2000). Four of these populations of plants are located in GU-C5 and one is in GU-C7.

The choice of these populations and municipalities was due to ease of access and native knowledge about the occurrence of 'do-sono' passion fruit in the region. Local communities make use of the fruit in their diets, and/or make the fruit available for commercialization in markets, for example CEASA in Vitória da Conquista, Bahia.

The number of fruits collected for each population was unequal, due to the variation of availability of the same (population) at the time of collection. The fruits were collected when they were completely ripe, having fallen to the ground in the morning. The particularity of collecting fallen fruit below the dossel of vigorous plants in the process of fruiting, which overlap their vines, impeded counting the number of fruits per plant, or even the number of plants evaluated in each population. Georeferencing data of the collection locations and number of fruits per population are found in Table 2.

After being collected, the fruits were packed in plastic bags, marked and transported for washing and manual de-pulping at the Plant Genetics Laboratory at the Vitória da Conquista campus of the Universidade Estadual do Sudoeste da Bahia [*State University of Southwest Bahia*] (UESB).

### Physicochemical characterization of the fruit

The following physical and chemical variables of the ripe fruits were measured: fruit weight (FW) in g; conformity index (CI = LD/TD,

**Table 2.** Populations of 'do-sono' passion fruit (*Passiflora setacea* DC) sampled in five populations, with data on collection location, sample size, geographic coordinates and altitude of collection location.

| Population | Location | Total Number of fruits | Number of fruits post-outlier detection | Longitude (West) | Latitude (South) | Altitude (m) |
|---|---|---|---|---|---|---|
| VC | Vitória da Conquista | 112 | 104 | 42° 19' 49.1" | 15° 05' 30.3" | 999 |
| MO | Mortugaba | 14 | 14 | 40° 48' 12.3" | 14° 53' 05.4" | 1015 |
| CO | Cordeiros | 17 | 17 | 41° 57' 00.8" | 14° 55' 09.7" | 704 |
| CD | Condeúba | 20 | 18 | 41° 58' 38.5" | 14° 52' 14.7" | 739 |
| PP | Piripá | 25 | 22 | 42° 05' 18.4" | 14° 40' 28.0" | 820 |

where, LD = lateral diameter and TD = transversal diameter), pulp weight (PW) in g; pulp yield [PY (%) = FW - SW/FW x 100, where SW = shell weight); shell thickness (ST) in mm; pH; total soluble solid level (TSS); citric acid percent (CA%) and proportion of soluble solids to acidity (TSS/CA%). The measurement of the variables 'proportion of total soluble solids' and citric acid percent were carried out according to Cardoso-Silva et al. (2007). The weight measurements in grams were obtained using a digital scale, and the dimensions in millimeters were obtained with a digital caliper.

#### Uni- and multivariate analysis

Prior to performing uni- and multivariate statistical procedures, the existence of extreme values was determined based on the Outliers-base function in the deviances (α = 0.05) BioEstat 4.0 (Ayres et al., 2005). The remaining quantity of fruits in the population after removal of extremes is found in Table 2.

The measurements of each physicochemical variables of fruit were submitted to unbalanced (α = 0.01) variance analysis (ANAVA) considering an entirely random statistical delineation with five treatments (populations) and fruits per plant used as repetitions. The ANAVA was carried out using BioEstat 4.0 software (Ayres et al., 2005).

The set of new measurements of the variables was submitted to unbalanced multivariate analysis of variance (Manova; α = 0.01), now considered a 'source of variation' the (*i*) two distinct ecogeographical regions (Pillai's trace, α = 0.01), or (ii) the five wild populations, independent of ecogeographical origin (Pillai's trace, α = 0.01).

The significance of the contrasts between location pairs was obtained via a Hotelling T-squared test (α = 0.01) (Kramer, 1972), which expressed the geographic distance between the populations. The MANOVA was carried out using the MINITAB$^{TM}$ (Minitab Inc., 1999) software.

#### Grouping and Mantel analyses

The variables were then submitted to grouping analysis (UPGMA) through calculation of the average euclidean distance between the populations, followed by adjustment between the distance matrix and the dendogram, employing the cophenetic correlation coefficient (*r)*.

Finally, analysis of the spatial variation standard was done in a multivariate context via Mantel analysis (1967), with the geographic distance matrix and the average euclidean distance being used to measure dissimilarity between the populations, with 1000 simulations. Both the grouping analysis used and the Mantel test were performed using the Genes program (Cruz, 2001).

## RESULTS AND DISCUSSION

ANAVA has identified that the five plant populations exhibit fruits that are statistically different in regard to physicochemical characteristics of fruit studied (*p* <0.0001), which denotes differential performance between the populations, and relates the existence of genetic variability sufficient to indicate priority areas for the purpose of collecting and selecting of the best populations of 'do-sono' passion fruit. The average values of the characteristics are presented in Table 3.

The morpho-agronomic descriptors were used to determine the genetic variability of the species obtained values of the variation coefficient (VC, %), which varied from 5.5 to 31.8; showing that the variables used present different levels of heterogeneity.

Even in one species, in each of the studied areas, the plants are subject to fluctuations of temperature, rainfall levels and other variants that may influence certain aspects of its genetic composition. That is, the mean may be adequate to express determined characteristics that do not appear in another location (Botezelli et al., 2000), it is what may be reported in the results obtained in the five studied populations.

Analyses of the fruits by collection area identified that the populations from Vitória da Conquista and Condeúba stand out for average values of physicochemical characteristics of fruit being adequate for industry, principally in regard to pulp yield and total soluble solids, and the areas are indicated as adequate for prospecting superior genotypes that may compos germplasm banks for pre-improvement studies and conservation of the species.

Significant multivariate genetic divergences have detected between the two distinct ecogeographical regions (Pillai's trace, *p value* < 0.001). That is, the geoenvironmental units C5 and C7 show natural populations of 'do-sono' passion fruit that genetically diverge among themselves, corresponding to the set of physicochemical characteristics of fruit evaluated.

Each of the five wild populations were tested in MANOVA independent of ecogeographical origin. The *p value* surveyed by the statistic from the Pillai's trace test (< 0.001) corroborates that these genetically diverge with

**Table 3.** Results obtained from the physicochemical analysis of five populations of 'do-sono' passion fruit.

| Population[1] | Fruit weight (FW) | Conformity index (CI) | Pulp weight (PW) | Pulp yield (PY) | Shell thickness (ST) | pH | Total soluble solid level | Citric Acid (%) | Proportion of soluble solids to acidity (TSS/CA) |
|---|---|---|---|---|---|---|---|---|---|
| VC | 48.48 | 1.27 | 27.18 | 55.51 | 3.29 | 3.21 | 15.56 | 2.04 | 7.92 |
| MO | 33.60 | 1.14 | 15.18 | 43.99 | 2.38 | 3.40 | 15.22 | 2.35 | 6.49 |
| CO | 40.63 | 1.13 | 20.84 | 49.73 | 4.92 | 2.94 | 13.71 | 2.71 | 5.60 |
| CD | 42.37 | 1.20 | 20.93 | 48.85 | 3.47 | 3.33 | 11.90 | 3.46 | 4.10 |
| PP | 55.41 | 1.10 | 27.42 | 50.40 | 5.37 | 3.02 | 14.39 | 2.54 | 6.52 |
| QMT | 0.0014 | 0.20 | 663.39 | 631.12 | 34.79 | 0.69 | 65.19 | 9.61 | 76.57 |
| QMR | 234.74 | 0.03 | 92.20 | 83.65 | 1.56 | 0.07 | 6.83 | 0.83 | 6.84 |
| X[2] | 44.10 | 1.17 | 22.31 | 49.70 | 3.89 | 3.18 | 14.16 | 2.62 | 6.13 |
| CV(%)[3] | 18.70 | 5.5 | 22.95 | 8.28 | 31.77 | 6.11 | 10.28 | 20.19 | 22.93 |

[1] The meaning of the abbreviations of the populations is found in Table 2; [2] General average; [3] variation coefficient, in percentage.

**Table 4**. Values from the Hotelling $T^2$ multivariate and their respective *p-value*, among five wild populations of 'do-sono' passion fruit (*P. setacea* DC) belonging to the geoenvironmental units C5 and C7.

| Population | VC | MO | CO | CD | PP |
|---|---|---|---|---|---|
| VC[1] | - | 63.84** | 122.85** | 299.7** | 86.62** |
| MO | | | 73.92** | 65.55** | 134.46** |
| CO | | | | 83.98** | 21.3* |
| CD | | | | | 134.54** |
| PP | | | | | - |

Note.: [1] The meaning of the abbreviations of the populations is found in Table 2; ** *p-value* < 0.0001; * *p-value* = 0.043

each other, with at least one contrast pair existing among the populations with multivariate differences. The Hotelling $T^2$ test ($\alpha = 0.01$) was used to test the two-to-two contrasts; values surveyed from the test, and their degree of corresponding statistical significance, are found in Table 4.

With the exception of the multivariate contrast between the pair of populations of PP and CO (*p value* = 0.043), a significant multivariate difference was detected (*p value* < 0.0001) for all of the other contrasts. It is worth noting that the geographic distance between populations PP and CO was the lowest among all of the contrast pairs (6 km) (Table 5). The Hotelling $T^2$ statistic corroborates the genetic differences detected by the individual ANAVAs. With the existence of differences among the vectors of average for the populations proven with the Hotelling $T^2$ test, the average euclidean distances of the five populations were calculated (Table 5).

In regard to genetic differences, populations CO and PP showed the lowest euclidean distance values (0.82), and the highest values were found for populations VC and CD (1.74). The non-contrast between CO and PP may be explained by the fact that both of these populations are

**Table 5.** Distance matrix among the five populations of 'do-sono' passion fruit plant (*P. setacea* DC), based on the measurement of nine physicochemical characteristics of fruit.

| Population[1] | VC | MO | CO | CD |
|---|---|---|---|---|
| VC | - | | | |
| MO | 1.61 | - | | |
| CO | 1.44 | 1.31 | - | |
| CD | 1.74 | 1.41 | 1.12 | - |
| PP | 1.28 | 1.68 | 0.82 | 1.49 |

[1]The meaning of the abbreviations of the populations is found in Table 2.

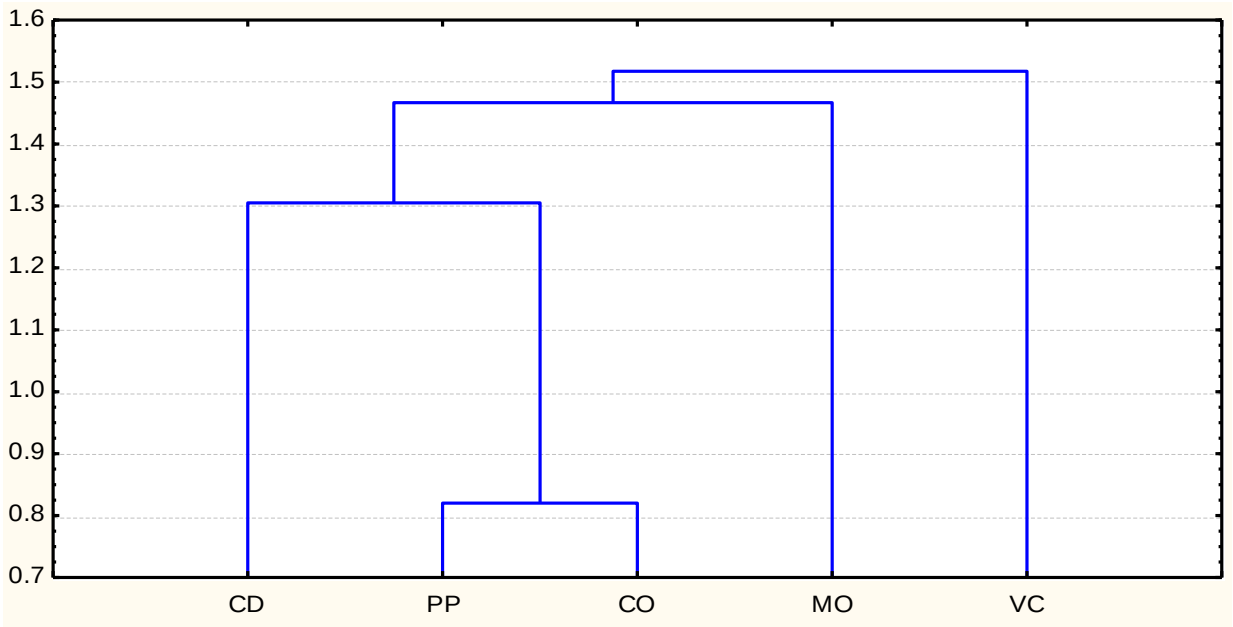


**Figure 1.** Dendogram of dissimilarity based on the average euclidean distance from the beginning of nine physical and chemical characteristics of fruit from five wild populations of 'do-sono' passion fruit (P. setacea DC), occurring in two geoenvironmental units in Bahia. The cophenetic correlation coefficient for the dendogram was 0.786.

found in the same geoenvironmental unit (GU-7), which shows more homogenous edaphoclimatic conditions, as well as due to the close physical proximity of these populations. The plants from CO and PP are more closely related as a result of the occasional gene flow between these two populations.

The greater genetic divergence found between populations VC and CD may be explained by ecogeographic origin. The two populations are located in distinct GUs [C5 (the population of Vitória da Conquista) and C7 (population of Condeúba)]. Considering this approach, note that the greatest genetic differences were related to the different geoenvironmental units, which can probably be explained by the greater geographic variability found, which may be associated with differences in soil type, elevation and precipitation. Based on these distances the hierarchical grouping was generated using the UPGMA method (Figure 1), which validates the results of the multivariate Hotelling $T^2$ test.

The formation of two groups can be noted. In the first group, the population of Vitória da Conquista is ordered in an isolated manner. It should be emphasized that this population is the only one among the five studied in this work pertaining to GU-C5. The second group was made up of populations from Mortugaba and a subgroup containing the populations from Condeúba, Cordeiros and Piripá (Figure 1). All four of these populations are in the UG-C7 (Figure 1).

The standards of similarity between the populations surveyed by the UPGMA may be analytically understood through the use of the simple Mantel test. The correlation coefficient between the genetic distance matrix and the

geographic distance showed a positive yet insignificant value ($r$ = 0.35; $p$ > 0.05). The structure in the space presented should not have a direct influence on the geographic distribution on these characteristics. The phenotypes of the populations may be influenced by other factors such as soil type, elevation, pluviometer, etc.; they are found independent of spatial distribution and genetic divergence, which indicates a possible adaptive process or response by a reaction standard to the environmental variations in these populations (Morales, 2000).

Finally, the use of the strategy of ecogeographical prospecting (Maxted et al., 1995) is an important tool for research of functional biodiversity prospecting of native passion fruit, similar to this study, in which genotypic variants presenting different magnitudes of physicochemical characteristics of fruit may be ecogeographically located and defined, for later use under the scope of pre-improvement conservation.

## Conclusion

For the majority of physical and chemical characteristics of the fruit, there was high phenotypic variability in the populations of 'do-sono' passion fruit studied; indicating that genetic gain through selection is possible.

The locations of Vitória da Conquista and Piripá are recommended for prospecting plants with fruit adequate for industry, principally due to pulp yield and proportion of total soluble solids.

The phenotypes of the populations of 'do-sono' passion fruit are probably influenced by the ecogeographic conditions where they are found, independent of distribution in space and their genetic differentiation, since the Mantel test was not significant, suggesting that an adaptive process exists that is playing on the environmental variations in these populations.

The adoption of consumption at the regional level endows the 'do-sono' passion fruit plant, which occurs naturally in the biomes of the Brazilian *caatinga* and *cerrado*, with the potential for future genetic domestication, with the goal of synthesis and selection of regional commercial varieties.

## Conflict of Interests

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

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*Full Length Research Paper*

# Antimicrobial susceptibility assessment of compound from *Aspergillus fumigatus*

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**Ethyl acetate extract of the culture filtrate *of Aspergillus fumigatus* on chromatographic analysis has led to the isolation of the compound, AF-1 which exhibited a significant *in vitro* antimicrobial activity against the tested pathogenic microorganism. The structure of the isolated compound, AF-1 was identified as 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one from its IR, UV, EI-MS, $^{13}$C-NMR, $^{1}$H-NMR, HMBC and HMQC data. This is the first report of the compound, AF-1 from the *A. fumigatus.***

**Key words:** *Aspergillus,* 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one, antimicrobial screening, bioactive compound.

## INTRODUCTION

Ever since mankind started suffering from ailments, the quest for finding remedies to treat the diseases started. The science of antibiotics has remained and will remain for many years, one of the most interesting natural sciences, in both theoretical and practical aspects. Determining new molecules which are potent bioactive component from newer sources to fight against pathogens is of great interest now-a-days. Fungi have proved to be capable of biosynthesizing secondary metabolites bearing conspicuous structural diversity, which could be further enlarged by structural modifications (Czarnik, 1996).

Fungal secondary metabolites are gaining importance in Pharmaceutical and Biotechnological applications. At present, at least six different components were commonly prescribed as medications isolated from *Aspergillus* origin (Varga and Toth, 2003). *Aspergillus* strains have been reported to be producers of metabolites with broad range of biological activities (Cheng-Bin et al., 1997). During recent decades, bacteria resistance to most clinically available antimicrobial agents has emerged at an alarming rate (Wu et al., 1999; Arias and Murray, 2009). Consequently, discovery of new antimicrobials has become increasingly crucial.

As a part of continuing research on the microbial metabolites from the soil samples we have isolated a number of antagonistic microorganisms, among the isolates selected one was identified by morphological and other criteria. Finally, we screened for the production of antimicrobial and pharmacological

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active compound.

## MATERIALS AND METHODS

### Isolation and identification of antagonistic microoganisms from soil samples

In a systematic screening program for the isolation of bioactive compound from microorganism, soil samples were collected into sterile boiling tubes with a sterile spatula near to the Rajshahi University Campus. Care was taken to see that the points of collection had widely varying characteristics as possible with regard to the organic matter, moisture content, particle size and color of soil (Reiner, 1982; Mythili and Das, 2011).

About 1 g of sample was transferred to a sterile flask containing 50 ml sterile water. The flasks were shaken on rotary shaker for 30 min and were kept aside for 30 min to settle down the particulate matter. The clear supernatant was diluted with sterile water. These dilutions (1/10 - 1/1000) were used as inocula. One ml of each of these dilutions were pipetted out into the medium, plated into petridishes 10 cm diameters and incubated at 28°C for three to seven days. For the isolation of antagonistic microorganisms from the above mentioned samples, the following media were used: starch casein agar medium, Czapek Dox agar, Czapek yeast agar and malt extract agar medium.

For the identification of the selected organism we emphasize the morphological methods including; macroscopic and microscopic characteristics according to Klich (2003) and McClenny's (2005) Identification Aspergillus Species. Finally we compared the morphological characteristics of tested *Aspergillus* species with those of standard *Aspergillus fumigatus*.

### Production, isolation and purification of the compounds

The organism was allowed to grow in a number of culture flasks of 500 ml capacity containing Czapek-Dox broth acidic medium at 35°C. The broth was separated from the mycelial mat on $8^{th}$ day to get the maximum yield of antimicrobial activity (data not shown). The culture filtrate was then subjected to repeated ethyl acetate extraction and the extract was evaporated under reduced pressure. The crude extract was resolved by thin layer chromatography (TLC), preparative TLC (PTLC) (Stahl, 1969) and obtained on large scale on column chromatography (CC) (Beckett and Stenlake, 1986). For checking purity of the compound, TLC was carried out using pre-coated silica gel 60 $F_{254}$ plates (Mercks) and detection was made by visualization under UV light (254 nm) and spraying with 0.1% vanillin sulfate spray reagent followed by heating.

### Spectral measurement

Ultra-violet (UV) spectra were recorded on a Beckman double beam spectrometer. IR spectra were obtained by a Perkin Elemer 1600 FTIR spectrometer. $^{1}$H-NMR (500MHz) and $^{13}$C-NMR (125 MHz) spectra were acquired on a JEOL JNM alpha spectrometer using TMS as internal standard. Electro spray ionization mass (EI-MS) was recorded on a JEOL DX-300 spectrometer. $^{1}$H-$^{13}$C HMBC and HMQC were recorded at 500 MHZ (proton) and 125 MHZ (carbon), respectively.

### Antimicrobial screening

The disc diffusion method (Radovanović et al., 2009) was used to test antimicrobial activity against 15 bacteria and three fungi. Solutions of known concentration (µg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of ethyl acetate. Dried and sterilized filter paper discs (7 mm diameter) were then impregnated with known amounts of the test substances using micropipette. Discs containing the test material were placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic (Kanamycin 30 µg/disc) and blank discs (impregnated with solvents) were used as positive and negative control, respectively. These plates were then kept at low temperature (4°C) for 24 h to allow maximum diffusion. The plates were then incubated at 37°C for 24 h to allow maximum growth of the organisms. The antimicrobial activity of the test agent was determined by measuring the diameter of zone of inhibition expressed in millimeter. The experiment was carried out in triplicate and the mean of the readings were taken. Griseofulvin (20 µg/disc) was used as standard for antifungal activity. The MIC values of the compound were determined against *Shigella dysenteriae, Salmonella typhi, Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus* by serial dilution technique (Hammond and Lambert, 1998).

## RESULTS AND DISCUSSION

### Identification of the organism

The isolated organism was identified as *A. fumigatus* based upon the following morphological characteristics:

#### *Macroscopic feature*

Growth rate is rapid; the colony size can reach 3 ± 1 cm within a week when grown on Czapek-Dox agar at 25°C; growth occurring at temperatures as high as 40°C and survival were maintained at temperatures up to 60°C; the texture of colonies varies from wooly to cottony to granular; surface colony color is smoky gray to green and the reverse is yellow due to the diffusible pigment; and color of very mature colonies turn to slate gray while atypical colonies may remain white with slight conidiation.

#### *Microscopic appearance*

Conidial heads are in the form of compact columns in an undisturbed culture. The chains of conidia are borne directly on broadly clavate vesicles. The conidiophores are smooth-walled up to 300 µM long, and terminate in a dome-shaped vesicle with a diameter of 20-30 µM long; Hyphae are septate and hyaline; the species is uniseriate producing a closely compacted phialides with size ranging from 5-10 × 2-3 µM, and only occurring on the upper portion of the vesicle; and conidia are round to sub-globse, smooth to finely roughened, and with diameter size of 2.0 - 3.5 µM.

### Characterization of the compound

The compound was obtained as colorless crystalline needle having melting point of 153°C.

#### *EI-MS analysis*

In the mass spectrum the compound showed molecular

**Table 1.** $^{1}H$-$^{13}C$ correlation using HMQC (500 MHz, CD30D).

| $^{1}H$-NMR | $^{13}C$-NMR |
|---|---|
| 6.49 (1H, s, H-3) | 110.8 (C-3) |
| 7.95 (1H, s, H-6) | 141.0 (C-6) |
| 4.41 (2H, s, H-7) | 61.2 (C-7) |

**Table 2.** $^{1}H$-$^{13}C$ correlation using HMBC (500 MHz, CD30D).

| $^{1}H$-NMR data | $^{13}C$-NMR data |
|---|---|
| 6.49 (1H, s, H-3) | 61.2,147.4,170.4,176.8 |
| 7.95 (1H, s, H-6) | 147.4,170.4,176.8 |
| 4.41 (2H, s, H-7) | 110.8,170.4 |

**Table 3.** Antibacterial activity of the compound and kanamycin standard.

| Test organism | Strain No. | Compound (30 µg/disc) | Compound (60 µg/disc) | Kanamycin (30 µg/disc) | Crude extract (60 µg/disc) |
|---|---|---|---|---|---|
| **Gram negative** | | | | | |
| *Shigella dysenteriae* | AL-35587 | 16 | 33 | 33 | 20 |
| *Shigella shiga* | ATCC-26107 | 17 | 27 | 31 | 20 |
| *Shigella boydii* | AL-17313 | 14 | 28 | 30 | 16 |
| *Shigella sonnei* | AJ-8992 | 14 | 27 | 29 | 17 |
| *Shigella flexneri* | AL-30372 | 15 | 27 | 33 | 19 |
| *Escherichia coli* | FPFC-1407 | 17 | 30 | 31 | 23 |
| *Pseudomonas aeruginosa* | QL-147 | 14 | 26 | 26 | 20 |
| *Klebsiella* spp. | CRL | 17 | 28 | 25 | 22 |
| *Salmonella typhi* | CRL | 16 | 32 | 27 | 18 |
| **Gram positive** | | | | | |
| *Bacillus subtilis* | QL-40 | 15 | 28 | 29 | 29 |
| *Bacillus cereu* | QL-29 | 16 | 27 | 30 | 30 |
| *Bacillus megatrium* | QL-38 | 14 | 25 | 27 | 26 |
| *Sarcina lutea* | QL-166 | 14 | 22 | 25 | 25 |
| *Staphylococcus aureus* | ATCC-259233 | 10 | 17 | 26 | 22 |
| *Streptococcus-β- haemolyticus* | CRL | 10 | 16 | 27 | 20 |

ion peak at m/z 142 *(W)* and (M*+1) peak at *m/z* = 142, which correspond to the molecular formulae $C_6H_5 4_4$.

### *UV Spectra*

In UV spectrum the compound showed absorption maximum, at 242 nM, which indicates the presence of aromatic carbonyl compound.

### *IR Spectrum*

In IR spectrum, the compound showed absorption band at 1725 and 3620 $cm^{-1}$, these might be due to carbonyl and hydroxyl group, respectively. $^{1}H$-NMR (500 MHz, $CD_3OD$) δppm: 6.49(IH, s, H-3), 7.95 (1H, s, H-6), 4.41(2H, s, H-7); $^{13}C$-NMR (125 MHz, $CD_3OD$) δppm: 170.4 (C-2.), 110.8 (C-3), 176.6 (C-4), 147.4 (C-5), 141.0 (C-6) and 61.2 (C-7) (Table 1).

The compound AF-1 was elucidated as 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one, on the basis of UV, IR, EIMS, $^{1}H$-NMR, $^{13}C$-NMR, HMBC and HMQC spectra (Table 2); and comparing the pyran derivatives (Budavari, 2000; CIS Information Services, 2000 and Lide and Milne, 1996). The structure of the compound is shown in Figure 1.

### Antimicrobial activity

The ethyl acetate extract of the culture filtrate of *A. fumigatus* and compound showed significant antibacterial activity against pathogenic organisms at a concentration of 30 and 60 µg/disc (Table 3). However, the activity of

**Table 4.** Antifungal activity of the compound.

| Test fungus | Diameter of zone of inhibition (mm) | | | |
|---|---|---|---|---|
| | Compound (100 µg/disc) | Compound (200 µg/disc) | Griseofulvin (20 µg/disc) | Crude extract (200 µg/disc) |
| *Tinea pedis* | 11 | 15 | 19 | 14 |
| *Tinea corporis* | 10 | 15 | 20 | 13 |
| *Candida species* | 13 | 18 | 22 | 12 |

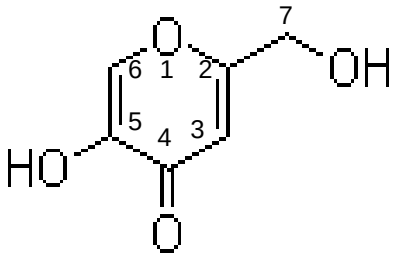


**Figure 1.** 5-Hydroxy-2-(hydroxymethyl)-4H-pyran-4-one.

the compound was potent against *S. dysenteriae, S. typhi* and *Escherichia coli* as compared to that of standard. The compound also possesses antifungal activity (Table 4). The antifungal activity was ten time less as compared to the standard (griseuofulvin).

## Minimum inhibitory concentration

The minimum inhibitory concentration of the compound against *S. dysenteriae, E. coli*, *S. typhi*, *B. subtilis* and *Sarcina lutea* were 64, 64, 128 and 256 µg/ml, respectively.

## Conclusions

The compound AF-1 (5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one) was the first report from *Aspergillus fumigatus*. The potency of antimicrobial activity of the compound was higher against gram-negative bacteria compared to gram-positive bacteria. Interestingly the antimicrobial activity of crude extract were different than the isolated pure compound, which reflect that the crude extract contained antimicrobial compound(s) other than the isolated compound and predominantly effective against gram-positive bacteria. The isolated compound AF-1 may be a very effective therapeutic agent, but further study is necessary for drug development.

## Conflict of Interests

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

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*Full Length Research Paper*

# Physiological properties of facultative and obligate alkalophilic *Bacillus sp.* strains isolated from Saudi Arabia

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**Isolation and identification of new alkalophilic *Bacillus* strains have increasing interest due to their possessing valuable and commercially interesting enzymes. To date, several researchers have studied the identification and characterization of alkalophilic *Bacillus* strains based on the phenotypic characterization as phylogenetic analysis of 16SrRNA. In the present study, six obligate and facultatively alkalophilic isolates were purified from dessert soil around Al-qunfotha city, in Saudi Arabia. All isolates were phenotypically and genotypically characterized. Among these isolates, AS3, AS4, AS5 and AS6 could grow at pH 9, 10, 11 and 12, but could not grow at pH 7 indicating that this isolates are obligate alkalophiles while, isolates AS1 and AS2 grew at pH range from 7 to 10, but could not grow at pH 11 and 12, suggesting that they could be facultative alkalophiles. All isolates could hydrolyze casein and starch, indicating that they possess interesting amylase and protease enzymes. Comparative sequence analysis of 16s rRNA of the six alkalophilic Bacillus strains indicated that these isolates share 99% identity with the previously isolated genes and belong to *Bacillus cohnii* at the full length gene nucleotide sequence level. The nucleotide sequences of 16SrRNA gene for the six isolates were given Gene-bank accession numbers: KP053301, KP053302, KP053303, KP053304, KP053305 and KP053306, respectively.**

**Key words:** *Bacillus cohnii,* 16SrRNA, obligate alkalophiles, facultative alkalophiles.

## INTRODUCTION

Genus *Bacillus* are more phenotypically heterogeneous than most other bacterial genera (Claus and Berkeley, 1986; Osman, 2012). There is a diverse group of *Bacillus* species living in highly alkaline terrestrial and aquatic environments. In the past decade there was a full revision of alkaliphilic *Bacillus* classification according to their phylogenetic and phenotypic characteristics (Takami and Horikoshi, 2000; Osman et al., 2013). The alkaliphilic and nonalkaliphilic species of the genus *Bacillus* are difficult to identify by traditional methods based on phenotypic characteristics (Woese, 1987; EL-Ghareeb et al., 2102). All the morphological and physiological characteristics of the native strains indicated that these isolates were from genus *Bacillus*. Native alkaliphilic *Bacillus* isolates are

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similar according to the biochemical characteristics. Some native isolates did not utilize much carbohydrate for growth, and no glucose. As a result, it was determined that the conventional tests based on phenotypic characteristics were insufficient for the differentiation of native alkaliphilic *Bacillus* isolates. Bacteria are widely distributed in nature. Most of the known microorganisms grow best at around neutral pH. However, there are bacteria that can grow in extreme alkaline environments (Muntyan et al., 2005). They are classified into two groups: alkalophilic bacteria, which are able to grow at pH above 10 and their optimal growth about pH 9 (Xu and Cote, 2003). The other group is called alkalotolerante bacteria, which show optimum growth at pH around 7, but able to grow at pH around 10 (Joung and Cote, 2002). Alkalophilic bacteria can be further divided into obligate and facultative alkaliphilic (Marie et al., 2005). The former group shows optimal growth of pH around 10 and cannot grow at pH around seven; whereas, the latter group grows at pH 7 and around 10 with optimal growth at pH 10 or above (Schallmey et al., 2004).

Interest in alkalophilic bacteria has increased during the last few decays due to their applications in ecological, industrial and biotechnology fields. Alkalophilic microorganisms represent a challenge to the basis of Michell's chemiosmotic theory. It has been reported that the cytoplasmic pH for alkalophiles growth at 10 to 11 is in the range of 8 to 9 (Ashis and Sudhir, 2011). It has been demonstrated that alkalophilic bacteria have a reversed transmembrane proton gradient. Despite that, alkalophilic bacteria have a higher growth rate than those of neutrophils (Felske et al., 2003). Furthermore, facultative alkalophilic show higher growth rate at alkaline pH than at neutral pH. To date, several researchers have identified and characterized alkalophilic *Bacillus* strains using phenotypic characteristics, DNA-DNA relatedness data, and analysis of the 16SrRNA sequence (Assaeedi and Osman, 2012). Although, these methods have been used for the classification of alkalophilic *Bacillus* species, but the characterization of these microorganisms is considered complicated due to their slow growth and their extreme pH which interfere with the results of phenotypic tests. Alkalophilic bacteria that grow well at pH range of 10 to 11 are widely distributed throughout the world and have been isolated from a variety of ecosystems including soil (Guffanti et al., 1986). There are only a few reports on alkalophilic bacteria isolated from Saudi Arabia (Salama et al., 1993).

The objectives of this study were to isolate, characterize and identify alkalophilic bacteria from Al-qunfotha region at Kingdom of Saudi Arabia. Strains were characterized using phenotypic characteristics, 16SrRNA gene sequencing.

## MATERIALS AND METHODS

### Collection of soil samples

A total 50 soil samples were collected from the top few centimeters of different locations surrounding Al-qunfotha city located in the western region of Saudi Arabia. Samples were collected from 2 to 5 cm below the surface with a shovel (Horikoshi and Akiba, 1982). Samples were stored on ice until were transported to laboratory where they were stored at +4°C.

### Growth medium

The medium used in this study consists of 1% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% $K_2HPO_4$, 0.02% $MgSO_4$ $7H_2O$, 2% Agar, pH 10.5 (Horikoshi and Akiba, 1982). The solution of $Na_2CO_3$ was autoclaved separately and added to the medium.

### Isolation and screening of alkalophilic isolates

For isolation of alkalophilic strains, 1 g soil samples were suspended in 10 ml of sterilized $H_2O$ and 1 ml of soil suspension were then plated on M1 agar medium. Plates were incubated at 30°C for 48 h. Single colonies showing different morphologies were picked and re-streaked for 2 to 3 times on agar medium until single uniform colonies were obtained. Isolates were then stored in 20% glycerol at -80°C. The recipe for liquid media was the same as the composition of M1 medium but without addition of agar. Single colonies were inoculated on MI medium at pH10 and re-streaked several times for purity check. Six isolates were designated as AS1, AS2, AS3, AS4, AS5 and AS6. Growth on M1 broth medium with and without peptone was measured by determination of the optical density at 660 nm using a Hitach specterophotometer (type 124).

### Morphological and phenotypic characterization

Cells actively growing on nutrient agar plates (pH 7.0 and 9.0) were used for cell and colony morphology. The formation of spores was tested by using nutrient broth cultures of 18 to 24 h supplemented with 5 mg/L of $MnSO_4.4H_2O$ and observed under a phase contrast microscope. Temperature (20 to 60°C), pH (6 to 12), and salinity (2 to 10% NaCl) ranges for growth were tested in nutrient broth, and after 24 h of incubation at 37°C the optic density of the cells at 600 nm was measured. Physiological characterization tests including Gram staining; anaerobic growth; catalase and amylase activities; casein, citrate, starch, tyrosine, gelatin, and urea utilization; reduction of nitrate to nitrite; acid production from sugars; the methyl red test; the Voges Proskauer test; indole and $H_2S$ production; and susceptibility to lysozyme were carried out according to the methods of Murray et al. (1994).

### PCR of 16S rRNA gene sequencing

16S rRNA were performed according to El-Menofy et al. (2014) using Forward primer GF: 5'-AGTTTGACTCTGGCTCAG-'3 and reverse Primer GR: 5'-TACGGCTACCTTGTTACGACTT-'3. These primers were also used for PCR amplification of the 16S rRNA gene. Genomic DNA was isolated as described by Abulreesh et al. (2012). Cells from 5 ml overnight culture for each isolate were harvested. Cell pellets were rinsed with 200 µl of NET buffer (0.1 M NaCl, 50 mM EDTA, 10 mMTris-Cl, pH 8.0) and re-suspended in 200 µl of GET buffer (50 mM glucose,10 mM EDTA, 25 mMTris-Cl, pH 8.0). 0.001 ug of lysozyme was added and the mixture was incubated at 37°C for 3 h. Twenty microliter of 10 mg/ml proteinase K was then added and the mixture was incubated at 37°C for 1 h. One hundred microliter 10% SDS was then added, and the mixture was incubated at 37°C for 1 h. The mixture was extracted several times with phenol: chloroform: isoamyl alcohol (24 : 24 : 1, v/v) until the interface was clear. DNA was precipitated by adding 1/25 volume

of 5 M NaCl and 2.5 volumes of 95% chilled ethanol. The precipitated DNA was rinsed with 1 ml of ice cold 70% ethanol, air dried, and re-suspended in 30 µl of sterilized distilled water. Selection of primers (Invitrogen, Paisley, UK) for PCR was according to Marchesi et al. (1998). The PCR reaction mixture (50 µl total volume) contained 200 µM of each dNTP, 0.5 for each µM primer, 10 mM trisHCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mMKCl, 2.5 U Taq polymerase (ABgene, Surry, UK) and 100 ng of template DNA. DNA amplification using primer was performed at the following temperature cycle: denaturation at 94°C for 2 min, 30 cycles at 94°C for 60 s, 50°C for 60 s, and 72°C for 90 s, final extension at 72°C for 7 min, respectively. A total of 10 µl of PCR products were analyzed by 1% agarose gel (Bioline, London, UK) electrophoresis and made visible by ethidium bromide (0.5 mg/ml) staining and ultraviolet (UV) transillumination. Sequencing of PCR products was performed by the research team of the biotechnology lab company, Cairo, Egypt; following the procedure described by (Sanger et al., 1977; Assaeedi et al., 2011). The deduced sequence was subjected to blast search tool from the national center of biotechnology, Bethesda, MD, USA (http://www.ncbi.nlm.nih.gov) the full length 16SrRNA sequence was aligned with reference homologues DNA sequence from NBCI database using multiple sequence alignment program of MEGA4. Phylogenetic trees were constructed by distance matrix based cluster algorithms viz weighted pair group with average (UPGMAs) (Saitou, 1987). *Bacillus cohnii* APT5 been used as a reference group and *Escherichia coli* as out-group.

## RESULTS AND DISCUSSION

### Isolation and screening of alkalophilic microorganisms

A total of six alkalophilic bacterial isolates were isolated from soil samples collected from Al-qunfotha area, Saudi Arabia. All strains were purified as a single colony and microscopically investigated. For routine work, all strain was grown in nutrient agar plate and kept at 4°C as well as they were grown in nutrient broth and stored in 20% glycerol under -80°C.

### Phenotypic characterization

Colonies of all six isolates are creamy white when grown on alkaline peptone medium. All six isolates were gram positive, motile rods, sub-terminal to terminal ellipsoidal spore in swollen sporangia. As presented in Table 1, isolates AS3, AS 4, AS 5 and AS 6 could grow at pH 9, 10, 11 and 12 with 9, but could not grow at pH7, indicating that this isolates are obligate alkalophiles while isolates AS 1 and AS 2 grew at pH ranged from 7 to 10, but could not grow at pH 11 and 12, suggesting that they could be facultative alkalophiles. In this context, it was reported that alkalophilic microorganisms constitute a diverse group that thrives in highly alkaline environments. They have been further categorized into two broad groups, namely, alkalophiles and alkalotolerants. The term alkalophiles is used for those organisms that were capable of growth above pH 10, with an optimal growth around pH 9, and are unable to grow at pH 7 or less. On the other hand, alkalotolerant organisms are capable of growing at pH values in excess of 10, but have an optimal growth rate nearer to neutrality (Guffanti et al., 1986). The extreme alkalophiles have been further subdivided into two groups, namely, facultative and obligate alkalophiles. Facultative alkalophiles have optimal growth at pH 10 or above but can grow well at neutrality, while obligate alkalophiles fail to grow at neutrality (Guffanti et al., 1986). All isolates could grow at 2% NaCl but could not grow at 5 and 10%. Only strain AS3 appeared as halotolerant, whereas, it could grow at 5% NaCl. All isolates grew at 45°C, but no growth was observed at 50°C. Negative reactions for all strains were recorded for lysis by KOH. The data in Table 1 shows that most of these isolates utilize a wide range of carbon sources including maltose, D-fructose, D-glucose, Sucrose and D-mannitol, but was not able to ferment lactose, D-xylose, raffinose, D-galactose, D-sorbitol or L-arabinose. Casein, gelatin, starch, citrate utilizations and amylase and catalase activities were all positive, but urea and tyrosine could not be utilized. Also, they were able to reduce nitrate to nitrite, but gas production was not observed from nitrate. The methyl red and Voges-Proskauer tests were negative for all isolates and indole and $H_2S$ were not produced. Isolates AS1 and AS2 could not utilize maltose and mannitol. Since the soils in Al-qunfotha region of western of Saudi Arabia contain high concentrations of arsenic and sodium chloride and are highly alkaline (pH ranging from 8.9 to 9.9), we expected that the extreme environment of the Al-qunfotha would be a good location for the discovery of previously unidentified alkalitolerant, halotolerant, endospore-forming organisms that maybe of ecological and/or commercial interest. One of the most important and noteworthy features of many alkalophiles is their ability to modulate their environment. They can alkalinize neutral medium or acidify high alkaline medium to optimize external pH for growth. However, their internal pH is between pH 7 and 9, always lower than the external medium. Thus, alkalophilicity is maintained by these organisms through bioenergetic membrane properties and transport mechanisms, and does not necessarily rely on alkali-resistant intracellular enzymes (Guffanti et al., 1986).

However, *Bacillus* species are difficult to identify by traditional methods based on phenotypic characteristics (Woese, 1987). In past decades, there was a full revision of alkalophilic *Bacillus* classification according to their phenotypic characteristics (Fritze et al., 1990). Sequence analysis of a 16s rRNA hyper-variant region has been a widely accepted technique (Saitou, 1987), and was reported to be a useful tool in the discrimination between the species in the *Bacillus* group (Jill, 2004). So far, genetic methods used in the characterization of alkaliphilic *Bacillus* have included 16s rRNA sequence data analysis (Nielsen et al., 1995).

Generally, when discriminating between closely related

**Table 1.** Phenotypic and biochemical characterization of alkalophilic isolates isolated from soil samples collected from Al-qunfotha area, KSA.

| Character | Alkalophilic Strains | | | | | |
|---|---|---|---|---|---|---|
| | AS1 | AS2 | AS3 | AS4 | AS5 | AS6 |
| Colony morphology | White , creamy | | | | | |
| Cell morphology | Rod | | | | | |
| Gram stain | + | + | + | + | + | + |
| Sporulation | + | + | + | + | + | + |
| Oxidase | + | + | + | + | + | + |
| Catalase | + | + | + | + | + | + |
| **Carbon source utilization** | | | | | | |
| D-Glucose | + | + | + | + | + | + |
| D-fructose | + | + | + | + | + | + |
| D-mannitol | - | - | + | + | + | + |
| Sucrose | + | + | + | + | + | + |
| Maltose | - | - | + | + | + | + |
| L-arabinose | - | - | - | - | - | - |
| D-glactose | - | - | - | - | - | - |
| D-sorbitol | - | - | - | - | - | - |
| D-xylose | - | - | - | - | - | - |
| D-raffinose | - | - | - | - | - | - |
| Tween 20 | + | + | + | + | + | + |
| Tween 40 | + | + | + | + | + | + |
| Tween 80 | + | + | + | + | + | + |
| D-lactose | - | - | - | - | - | - |
| **Hydrolysis of** | | | | | | |
| Gelatin | + | + | + | + | + | + |
| Casein | + | + | + | + | + | + |
| Starch | + | + | + | + | + | + |
| Urea | - | - | - | - | - | - |
| **Other biochemical tests** | | | | | | |
| Reduction of Nitrate to nitrite | + | + | + | + | + | + |
| Voges and Proskaur reaction | - | - | - | - | - | - |
| Growth at pH 7 | + | + | - | - | - | - |
| 8 | + | + | + | + | + | + |
| 9 | + | + | + | + | + | + |
| 10 | - | - | + | + | + | + |
| 12 | - | - | + | + | + | + |
| Growth at NaCl 2% | + | + | + | + | + | + |
| 5% | - | - | | - | - | - |
| 10% | - | - | - | - | - | - |
| Growth at (°C) :30 | + | + | + | + | + | + |
| 40 | + | + | + | + | + | + |
| 50 | - | - | - | - | - | - |

species of the same genus, DNA-DNA hybridization, as well as housekeeping genes sequences should be the methods of choice, in accordance with the proposed molecular definition of species (Berkum et al., 1996). Since, all isolates that were identified in this study are closely related to *B. cohnii,* thus, our results indicate that

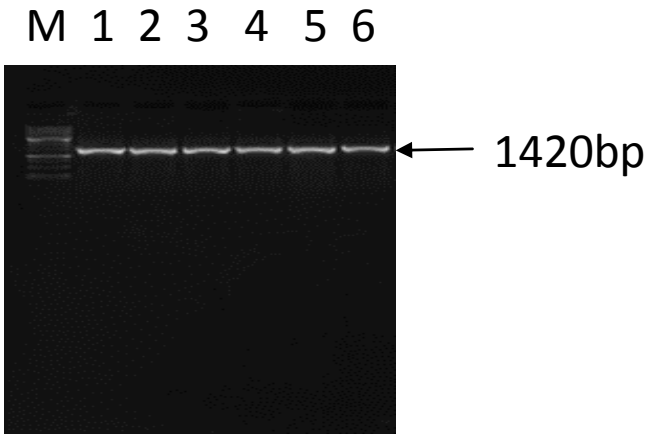


**Figure 1.** Agarose gel electrophoresis of PCR products of the 16S rRNA fragments for isolates number 1 to 6 (AS1, AS2, AS3, AS4, AS5 and AS6 respectively). M, 1 Kb DNA ladder marker.

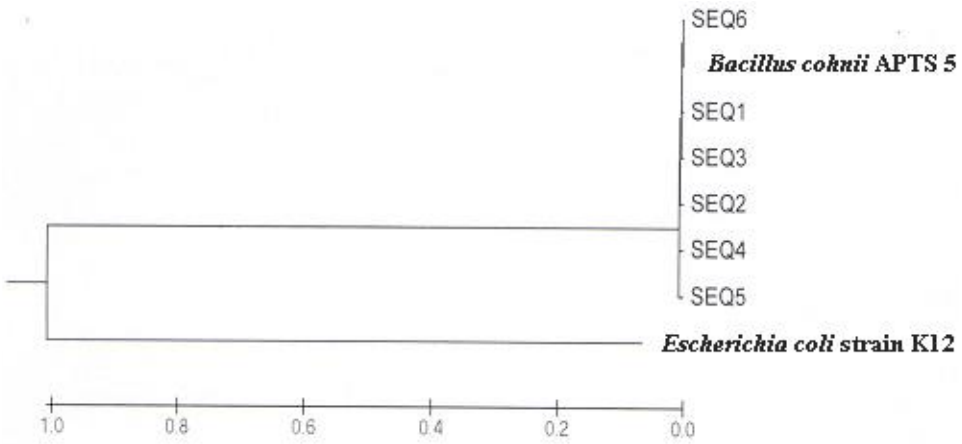


**Figure 2.** Phylogenetic relationship of the six isolates based on 16s rRNA sequence. SEQ1 to 6 represent isolates AS1, AS2, AS3, AS4, AS5 and AS6 respectively.

*B. cohnii* occurred in Al-qunfotha area. As reported by Assaeedi and Osman (2012), all alkalophilic bacteria isolated so far showed no growth in the absence of sodium ions at high pH value. This is due to the presence of acetate inside at pH 10. Therefore as found in the present study alkalkophilic strains use sodium ions to drive the solute uptake. From these results, it could be suggested that AS1 and AS2 isolates were facultative alkalophilic, while isolates AS3, AS4, AS5 and AS6 were obligate alkalophiles. Overall, the results obtained in this study suggest that a variety of alkalophilic and alkalitolerant, endospore- forming bacteria occurred and inhabit the Al-qunfotha area, KSA. However, further work such as isolation and characterization of the interested alkalophilic enzymes from these strains are in progress.

PCR of 16S rRNA gene sequencing and phylogenetic analysis. To identify the taxonomy of alkalophilic isolates, DNA was isolated and PCR amplification of the 16S rRNA was performed using primer. Primer was able to amplify a 1420 bp fragment (Figure 1). Homologs of the deduced sequence were identified using BLAST and Gene Bank from the National Centre of Biotechnology, Bethesda, MD, USA (http://www.ncbi.nlm.nih.gov). The partial 16S rRNA gene sequence was aligned with reference homologous DNA sequences from Gene Bank using the multiple sequence alignment programs in MEGA4. Alignment by BLAST showed that the primers only targeted 16SRNA gene. The results reveal that the six alkalophilic isolates sharing 99% 16SrRNA gene sequence similarity are classified under the same species. The 16SrRNA based phylogenetic analysis demonstrated 99% sequence similarity with *B. chonii*, suggesting that, these six isolates belong to this species (Figure 2). The 16s rRNA sequence data showed that isolates number 1, 2 and 3 had high similarity with *B. cohnii* strain D7023, while isolates number 4, 5 and 6 had

high similarity with *B. cohnii* strain T-46. All sequence data were deposited into Gene Bank with accession numbers: KP053301, KP053302, KP053303, KP053304, KP053305 and KP053306 respectively, (Figure 2). Phylogenetic relationship of the six isolates was based on 16s rRNA sequence; the tree was generated using the neighbor-joining method and the sequence from *E. coli* strain 12 was consider as out group. The sequence of *B. cohnii* APTS 5 was used as reference strain.

## Conflict of Interests

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

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*Full Length Research Paper*

# Phytochemical screening and antimicrobial activities of the leaf extract of *Entandrophragama angolense*

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**The phytochemical screening and antimicrobial activities of the leaf extract of *Entandrophragma angolense* were investigated using the agar well diffusion method. The methanolic extracts at crude level were shown *in vitro* to inhibit *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli* and *Streptococcus cremoris* with diameter zones of inhibition ranging from 6 to 14 mm. Minimum inhibitory concentration was 3.13 and 6.25 mg/ml for Gram positive and negative bacterial strains, respectively. Minimum bactericidal concentration (MBC) was 6.25 and 12.5 mg/ml for Gram positive and negative bacterial strains, respectively. The leaf extract also had bioactive compounds such as tannins, alkaloids, saponin and cardiac glycoside which may be responsible for the biological properties of this plant. The study confirms the antibacterial potential of *E. angolense.***

**Key words:** *Entandrophragma angolense,* antimcrobial activity, phytocheamical.

## INTRODUCTION

*Entandrophragma angolense* (Welw) is a large forest tree recognized by its large fruits which split from the base. The bark too is distinctive as it is very smooth. *E. angolerise* is used as a folklore medicine: as an antimalaria, antiulcer, haematinic and for treatment of other gastrointestinal disorders. The antiulcer activity of the plant has been investigated and the compound responsible for this activity was found to be methyl angolensate. It is of interest to note that most plant extracts have been reported to possess inhibitory substances and lethal activities on some pathogenic microorganisms *in vitro* (Oluma and Elaigwu, 2006).

Medicinal plants contain large varieties of chemical substances which possess important therapeutic properties that can be utilized in the treatment of human diseases (Vijaya and Ananthan, 2001). The studies of medicinal plants used in folklore remedies have attracted the attention of many scientists in finding solutions to the problems of multiple resistances to the existing synthetic antibiotics. Most of the synthetic antibiotics now available in the market have major setback due to the multiple resistance developed by pathogenic micro-organisms against these drugs. Thus, the need to search for new and more potent antimicrobial compounds of natural origin to combat the activities of these pathogens form the basis for this study.

## MATERIALS AND METHODS

The experimental plant leaves were collected from Ogbese, Ondo State, Nigeria and was identified in the Herbarium of Department of Biological sciences, University of Abuja, Abuja. FCT, Nigeria.

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**Table 1.** Phytochemical screening of the methanolic leaf extract of *E. angolense.*

| Bioactive constituent | Observation |
|---|---|
| Alkaloids | + |
| Saponins | + |
| Tannins | + |
| Phlobatanins | - |
| Anthraquinones | - |
| Cardiac glycosides | + |

+ = Present; - = absent.

**Table 2.** Means zone of inhibition of the methanolic leaf extract of *E. angolense* for organisms.

| Test microorganism | Extract at 100 mg/ml (mm) | Extract at 50 mg/ml (mm) | Chloramphenicol at 100 mg/ml (mm) | Chloramphenicol at 50 mg/ml (mm) |
|---|---|---|---|---|
| *Staphylococcus aureus* | 14.10 | 11.20 | 17.40 | 9.30 |
| *Streptococcus cremoris* | 11.30 | 9.20 | 13.00 | 8.40 |
| *Escherichia coli* | 12.40 | 10.80 | 15.50 | 10.20 |
| *Pseudomonas aeruginosa* | 9.70 | 7.80 | 14.50 | 10.40 |

**Extraction of leaves**

The procedure was as described by Odebiyi and Sofowora (1979). 100 g of the powdered leaves were extracted with 95% boiling methanol using a soxhlet extractor. The extract was filtered and evaporated to dryness using a rotary eraporator to give a dark green gummy residue.

**Phytochemical screening of extract**

This was as by Odebiyi and Sofowora (1979), Solomon-Wisdom et al. (2011); Solomon-Wisdom and Shittu (2010). The leaf extract was screened for saponin, alkaloids, tannins, phlobatanins anthroquinones and cardiac glycosides.

**Test organisms**

The test organisms namely *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* and *Streptococcus cremoris* were collected from Microbiology Laboratory of University of Abuja, Teaching Hospital, Gwagwalada Abuja, Nigeria. The stock cultures of bacteria were maintained on nutrient agar slants.

**Antibacterial assay**

This was as described by Odama et al. (1986). An aliquot of 0.1 ml of 1% barium chloride was added to 9.9 ml of $H_2SO_4$ to give a Mctarland turbidity standard suspension No 1. This turbidity approximates bacterial density of about $3x10^8$ organisms per ml. About 0.2 ml of the standardized suspension of the bacterium test growth in nutrient broth was pipetted into Muller Hinton Agar plates and the extract was used. The plates were incubated at 37°C for 24 h and the zones of inhibition were then measured to the nearest millimeter using a ruler (Erickson and Sherris, 1971). The minimum inhibitory concentration (MIC) was determined using the agar incorporated method as described by Abdulrahman (1986). This was done by using 0.2 ml of the standardized bacterial density of 3 x $10^8$ organisms per ml. The inoculums were pipetted on the Muller Hinton Agar incorporated with the extracts at various concentrations and incubated at 37°C for 24 h. Following the incubation, the growth of the organism on the agar plates with different concentration of the extracts were observed and measured.

## RESULTS AND DISCUSSION

The result of phytochemical screening shows the presence of alkaloids, saponins, tannins and cardiac glycosides, however, phlobatanins and anthraquinones are absent in the leaf extract.

The entire organisms were sensitive to the extract with Gram positive organisms having higher inhibitory zones with *S. aureus* having the highest zone inhibition of 14.10 mm at 100 mg/ml of the methanolic extract of *E. angolense* and for the Gram negative the highest zone of inhibition was recorded in *E. coli* (12.40 mm at 10 mg/ml), while the least zone of inhibition was recorded in *P. aeruginosa* which had the zone of inhibition of 7.80 mm at 100 mg/ml (Table 1).

The antibacterial activity results of *E. angolense* showed good effect against the Gram positive and negative microorganisms. Table 2 shows that MIC of methanolic leaf extracts of *E. angolense* for Gram positive organism is 3.13 mg/ml while for the Gram negative organism, it is 6.25 mg/ml.

Table 3 shows the MIC of the methanolic leaf extract of *E. angolense* against all organisms tested. MIC for the Gram positive organism was 3.13 mg/ml while for the

**Table 3.** Minimum inhibitory concentration of the methanolic leaf extract of *E. angolense*.

| Test microorganism | Concentration of extract (mg/ml) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 100 | 50 | 25 | 12.5 | 6.25 | 3.13 | 1.56 | 0.78 | 0.39 | 0.195 |
| *Staphylococcus aureus* | + | + | + | + | + | + | - | - | - | - |
| *Streptococcus cremoris* | + | + | + | + | + | + | - | - | - | - |
| *Escherichia coli* | + | + | + | + | + | - | - | - | - | - |
| *Pseudomonas aeruginosa* | + | + | + | + | + | - | - | - | - | - |

+ = Inhibition; - = no inhibition.

**Table 4.** Minimum bactericidal concentration of the methanolic leaf extract of *E. angolense*.

| Test microorganism | Concentration of extract (mg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | 100 | 50 | 25 | 12.5 | 6.25 | 3.13 |
| *Staphylococcus aureus* | + | + | + | + | + | - |
| *Streptococcus cremoris* | + | + | + | + | + | - |
| *Escherichia coli* | + | + | + | + | - | - |
| *Pseudomonas aeruginosa* | + | + | + | + | - | - |

+ = Inhibition; - = no inhibition.

Gram negative organism, it was 6.25 mg/ml. Table 4 shows the MBC of Gram positive organism to be 6.25 mg/ml and that of Gram negative to be 12.5 mg/ml.

Table 4 shows the minimum bactericidal concentration (MBC) of the methanolic leaf extract of *E. angolense* for both Gram positive and negative microorganisms; the MBC of *E. angolense* for Gram positive organisms was 6.25 mg/ml, while for Gram negative microorganisms (*E. coli* and *P. aeruginosa*), it was 12.5 mg/ml.

Phytochemicals have received increasing attention because of interesting new discoveries considering their biological activities (Solomon-Wisdom and Shittu, 2010). In most developing countries of the world, plants are the main medical sources used in treating infectious diseases. The various photochemical compounds detected are known to exhibit medicinal activity as well as physiological activity (Sofowora, 1992). Plants are important source of potentially useful structures for the development of new chemotherapeutic agent. They have an almost limitless ability to synthesize aromatic substances, most of which are phenols or other oxygen substituted derivatives (Geissman, 1963).

In the present study, methanolic extracts showed a great amount of phytochemicals which are of medicinal importance to human (Liu, 2004; Solomon-Wisdom et al., 2011). They are routinely used in medicine because of their profound biological activities. These compounds served as natural antibiotics, which help the body to fight infectious and microbial invasions (Sodipo et al., 2000, 2002; Aliyu et al., 2011; Shittu and Ogbaje, 2002). When the activity of the methanolic leaf extract was compared with that of the standard antibiotics at the same concentration, it was observed that the leaf extract compared favourably with those of standard antibiotics.

## Conclusion

The findings of the bioactivity of the leaf extract of *E. angolense* are beneficial as it indicates the emergence of new antibiotics with such a wide spectrum of activity. The results obtained show clear cut idea on the traditional uses of the plants. Plants that produce antimicrobials have enormous therapeutic potential as they serve the purpose with lesser side effects that are often associated with synthetic antimicrobials. Further research is however still necessary to determine the identity of the antibacterial compounds in these plant parts and also to determine their full spectrum of efficacy.

## Conflict of Interests

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

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*Full Length Research Paper*

# Enhancement of biomass production and nutrition utilization by strain *Lactobacillus acidophilus* DGK derived from serial subculturing in an aerobic environment

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**Successive subculturing under aerobic conditions for 14 days could be used to improve characteristics of a parent strain with a growth defect. Identification of the derived strain was based on the API 50 CHL test kit and partial sequencing of the *hsp60* gene. Results show that an aerotolerant strain, *Lactobacillus acidophilus* DGK, was created with improved nutrition utilization. Characteristics of shorter rod-shape, higher growth rate and higher level of lactic acid concentration were observed in the domesticated strain which produced higher biomass (4.54 g dry cell weight (DCW)/L) and viable counts (9.5 x $10^9$ CFU/mL) compared to the original strain which were 1.06 g DCW/L and 1.61 x $10^9$ CFU/mL, respectively under aerobic conditions. Utilization of carbon and nitrogen sources was significantly improved by the derived strain except the raffinose. These results indicate that serial subculturing is a plausible method for the generation of modified strains with enhancing nutrition utilization or improving various characteristics which were beneficial in industrial processes.**

**Key words:** Biomass; serial subculture; *Lactobacillus acidophilus; hsp60* gene.

## INTRODUCTION

Lactic acid bacteria (LAB) such as *Lactobacillus rhamnosus* GG (Wang et al., 2013; Soukoulis et al., 2014), *Lactobacillus bulgaricus* (Nagai et al., 2011; Ashraf and Shah, 2011), *Streptococcus thermophilus* (Iyer et al., 2010; Ashraf and Shah, 2011) or *Lactobacillus paracasei* 33 (Lin et al., 2014; Miao et al., 2014) have been extensively used in fermented milk or probiotic products for health benefit. Biomass production of these probiotics through fed-batch fermentation is important in industrial processes for cost reduction. During the technological processes for production of a probiotic product, factors such as medium formulation, stirring speed of blades, aeration rate, temperature and drying conditions may threaten the viability and activity of the bacteria which

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generally are microaerobic or anaerobic. Therefore, the development of effective strategies to improve certain characteristics, such as resistance to oxygen stress, bile salt and acid, which would allow adaptation to human gastrointestinal environments, and to survive the production process is of the utmost importance. These enhancements would increase cell growth and reduce the cost of commercialized probiotic products.

Most LAB strains lack catalase, cytochrome and heme-containing proteins (Chiancone and Ceci, 2010), which protect against oxidative stress or oxygen toxicity. Physiological adaptation to oxidative stress in aerobic microorganisms requires enzymatic defense against oxidative stress, examples of which include catalase, superoxide dismutase (SOD), glutathione reductase and peroxidase (Jänsch et al., 2011). NADH oxidase and iron-binding proteins were reported to correspond to oxygen tolerance or aerotolerance in Streptococcus mutants (Higuchi et al., 2000). Most of the studies on stress responses at physiological levels have focused on oxygen, temperature, acid and bile salt-adaptation processes (Sanz, 2007; Koponen et al., 2012). In one study, viability of the heat-adapted *Lactobacillus paracasei* was significantly enhanced to withstand heat stress during spray drying (Desmond et al., 2001). The approach to improve the survival of probiotic bacteria by stress adaptation has been based heavily on short exposures of viable cells to various sub-lethal stress factors to induce tolerance to subsequent lethal stress conditions (Saarela et al., 2004). The method of creating improved probiotic strains by acid stress adaptation treatments, resulting in improved stress tolerance and biological properties, has been reviewed previously (Sanz, 2007). Besides, cell length alternations and metabolic impacts derived from the stress reactions had been reported in lactobacilli (Serrazanetti et al., 2009; Krieger et al., 2013).

The strain Lactobacillus acidophilus GK isolated from healthy infant feces is sensitive to aerobic conditions. This impeded bacterial growth when incubated under aerobic conditions with a controlled pH and mild agitation at 60 to 150 rpm during fed-batch fermentation in preliminary experiments. This property has limited the production of L. acidophilus for probiotics. Prolonged exposure of LAB to preset conditions (such as acid) has been shown to be an effective strategy for generating derivative strains with a stable acid-resistant phenotype from acid-sensitive strains of species commonly found in the human gut (Andriantsoanirina et al., 2013). Therefore, the purpose of the present report was to develop L. acidophilus GK-derived strains with enhanced aerobic tolerance to adapt to the fermentation process and to compare the nutrition utilization between the domesticated strain and the parental strain. The results of this study will be important in the improvement of these strains' growth and functionality during aerobic fermentation, as well as to enable them to be stored as dried products for commercial use as probiotics.

## MATERIALS AND METHODS

### Microorganism, strain activation and medium composition

The LAB, *Lactobacillus acidophilus* GK, was provided by the Grape-King Company (Taiwan). This strain and its domesticated strain, *L. acidophilus* DGK, were cultured or subcultured in MRS broth supplemented with 0.05% cysteine (MRSC). Strains were maintained in a -80°C freezer, thawed at 37°C and incubated in MRSC broth at 37°C for 16 h or activated on an MRSC agar plate for 24 h. The medium compositions for carbon source or nitrogen source replacement are as follows: glucose, 10 g/L; yeast extract, 10 g/L; soy peptone (Scharlau), 10 g/L; sodium acetate, 5 g/L; ammonium citrate, 2 g/L; $MgSO_4.7H_2O$, 0.2 g/L; $MnSO_4.4H_2O$, 0.05 g/L and corn steep liquor 10 mL/L. Fermentation was performed in 10 mL of culture medium in a 50 mL Hinton flask with or without stirring at 150 rpm. The fresh culture (1%) was inoculated into fermentation medium and cultivated at 37°C for 24 h.

### Adaptation of the aerotolerant strain from *L. acidophilus* GK

A fresh culture of *L. acidophilus* GK (1%) was subcultured into another MRSC medium every day. The culture containing 10 mL of medium in a Hinton flask was incubated at 37°C for 24 h at 150 rpm. After 14 days of successive subculturing and incubation in this manor, the final culture medium was diluted, spread onto an MRSC agar plate and incubated at 37°C for approximately 24 to 36 h.

Population analysis was carried out for further screening of the derived strains that exhibited higher biomass production through observing the colony size and smaller cell size through microscope examination. More than 400 larger colonies, which were also hypothesized to have a higher growth rate than the original strain, were selected, compared and cultivated under aerobic conditions (as above) with shaking at 150 rpm. After 16 h incubation, cell density was measured at 600 nm, and the size of the cells in fermented culture was observed by microscopy. The colonies with higher density and reduced size were isolated and preserved in 10% glycerol at -80°C for later use. A domesticated strain possessing the desired characteristics of aerotolerance, reduced size and high biomass production was selected from the isolated cultures through the above screening procedure and designated as *L. acidophilus* DGK.

### Characteristics and identification of the domesticated strain *L. acidophilus* DGK

The differences in morphology, Gram-staining and acid/bile salt resistance were examined between the domesticated strain and the original one. For Gram-staining, a loop of fresh culture was spread onto a glass slice and then stained with crystal violet solution for 1 min. The slide was then washed, and iodine solution was added. Next, the slide was washed again with ethanol and stained with safranin for 30 s. After the stained slide air-dried, it was examined by microscopy. Cells with a deep blue color were identified as Gram-positive, while a red color indicated Gram-negative cells. To evaluate tolerance to acids and bile salts, 1 mL of fresh culture was mixed with 9 mL of phosphate-buffered saline (PBS) solution (pH 2 adjusted by HCl) and incubated at 37°C for 3 h with shaking at 80 rpm. Cell viability was determined by counting the number of colony forming units (CFU) after inoculation of serially diluted culture onto MRSC agar plates. The viable colony count for the control was obtained by serially diluting the cells with an acid-free PBS buffer control (pH 7.2) and counting the CFU on agar plates. Two-milliliter aliquots of the acid-treated samples (pH 2.0 PBS for 3 h) were centrifuged at 6000 rpm for 10 min to test for bile salt tolerance. The cell pellet was resuspended in pH 7.2 PBS and added to 10 mL of

MRSC broth containing 0.3% (W/V) of oxgall, and the cultures were incubated at 37°C for 24 h. Samples were taken at time intervals (3, 12 and 24 h) to determine CFU on MRSC agar plates. All cultures were performed in triplicate in 50 mL Erlenmeyer flasks containing 10 mL of the corresponding medium at 37°C for 24 h.

Biochemical tests were performed using an API 50 CHL test kit (BioMorieux Vitek, Inc., Hazelwood, MO, USA), according to the manufacturer's directions, to identify strains. A fresh culture (1 mL) was washed twice and resuspended in 1 mL of deionized water. The suspended culture was added to 5 mL of sterile water until the density matched the standard "1.0 McFarland Nephelometer" (PML Microbiologicals inc, USA). Two volumes of the diluted culture were poured into a 10 mL API 50 CHL cultivation medium for use as the seed culture. Each test well in the API 50 CHL was inoculated with 90 μL of the seed culture and incubated at 37°C for 24 or 48 h. The identification was carried out using the automated ATB Plus V2.6.8 analysis with the API 50 CHL version 5.0.

#### Amplification and sequencing of the partial *hsp60* gene

Oligonucleotide primers LB308F (TGAAGAAYGTNRYNGCYGG) and LB806RM (AANGTNCCVCGVATCTTGTT), previously described by Blaiotta et al. (2008), were used to amplify a 499-bp fragment internal to the *hsp60* gene. PCR amplifications were performed with a 25-μL total volume including 2 μL of the target DNA, 2 μL of each primer (25 μM), and 5.0 μL of 5 X Hot Start Mix II containing 0.75 U of Hot Start *Taq* DNA polymerase, 5 X reaction buffer, 10 mM $MgCl_2$ and 250 μM dNTP (Genemark, Taiwan). The PCR consisted of 35 cycles (20 s at 95°C, 30 s at 57°C and 30 s at 72°C) followed by a denaturing step at 95°C for 2 min and a final cycle at 72°C for 5 min. The PCR amplification fragments were resolved by agarose (2%, w/v) gel electrophoresis and visualized with SafeView DNA stain (Applied Biological Materials, Canada). After gel electrophoresis, the 499-bp PCR fragment was excised and purified by using a Micro-Elute DNA Clean/ Extraction kit (Genemark, Taiwan) and cloned by pOSI-T PCR cloning kit (Genemark, Taiwan). The recombinant pOSI-T plasmids were prepared and subjected to sequencing with the primer SP6 using an ABI Prism 377 DNA sequencer (Mission Biotech, Taipei, Taiwan). A search for DNA similarity was performed using the Blastn software (National Center for Biotechnology information, Bathesda, Maryland, USA).

#### Effects of carbohydrate and nitrogen sources on fermentation by the domesticated strain

The cultivation experiments were conducted using 50 mL Hinton flasks containing 10 mL of cultivation medium. The seed culture used as the inoculum was grown at 37°C for 16 h in a 10 mL test tube without shaking. The cultivation medium was inoculated with 1% (v/v) of the seed culture and incubated at 37°C for 24 h under either shaking (150 rpm) or static conditions. Various carbon and nitrogen sources were tested to examine the effects of medium compositions on cell growth and pH. Bacterial growth was measured as the absorbance of cell suspensions at 600 nm in a UV/VIS spectrophotometer (U-1800, Hitachi, Japan). The biomass was expressed as g/L of dry cells, based on a previously established regression between absorbance and cell dry weight. The supernatant was used for the analysis of glucose concentration. All of the experiments were performed in triplicate.

#### Analytical methods

The biomass of the fermented cultures was calculated by comparing the correlation curve between absorbance values of cell density ($OD_{600nm}$) and dry cell weight (g DCW/L). The pH was measured using a pH-meter SP-2300 (Suntex, Taiwan). The concentration of glucose in the supernatant was measured by the DNS method. Lactic acid was assayed by HPLC according to Andersson and Hedlund (1983).

#### Statistical analysis

Analyses of variance (ANOVAs) were performed on the data, and difference was considered significant at $p<0.05$.

## RESULTS

### Growth and characteristics of the domesticated strain *L. acidophilus* DGK

A fresh culture of *L. acidophilus* GK was successively subcultured into fresh MRSC medium every day and incubated with shaking (150 rpm), resulting in the adaptation of the strain to an aerobic environment. A population analysis of colonies selected after 14 days of culture was further applied to screen larger size of colonies, which might exhibit higher growth rate and biomass production. An aerotolerant strain was finally isolated and designated as *L. acidophilus* DGK. The appearance of the test tubes cultivating strains before and after adaptation was significantly different (Figure 1). *L. acidophilus* GK cells were concentrated at the bottom of the test tube, exhibiting the intrinsic characteristics of an anaerobic strain. In contrast, the domesticated strain, *L acidophilus* DGK, became aerotolerant, grew well and exhibited abundant biomass production, which would be beneficial for cell growth during fed-batch fermentation under agitation. Fermentation in bioreactors is generally performed under controlled conditions, which include monitoring temperature, pH, agitation and aeration rate. These conditions have been shown to affect LAB production significantly, with agitation being among the most important. The process of LAB biomass production in bioreactors occurs either by batch or fed-batch fermentation, in which stirring the medium is a critical step during the pH control and the feeding processes. In the present study, the growth of *L. acidophilus* GK was significantly reduced during pH control or feeding the concentrated medium due to agitation of the culture medium. The domesticated strain *L. acidophilus* DGK grew well in an aerobic environment and was suitable for cell proliferation during the fermentation process. NADH oxidase and superoxide dismutase (SOD) have been reported to play an important role in the regulation of aerobic metabolism in LAB (Higuchi et al., 2000). Therefore, it is possible that the aerotolerant strain *L. acidophilus* DGK might have higher activities of NADH oxidase or SOD when cultivated under aerobic conditions.

The cellular size of the domesticated strain was smaller than the parental strain, as shown in Figure 1. The cell density of the fermented culture by the domesticated strain (4.81 g DCW/L) was higher than that of the original

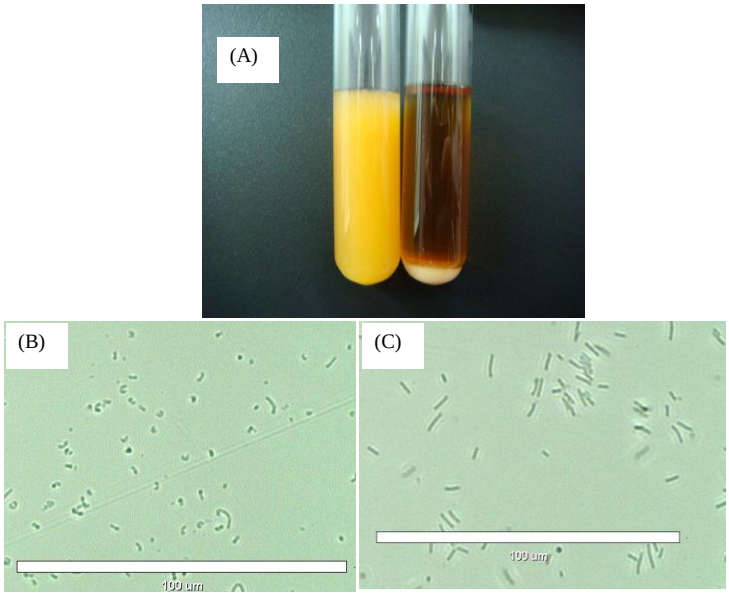


**Figure 1.** Appearance of cell cultures cultivated in MRSC for 16 h in test tube; left tube, the domesticated strain *L. acidophilus* DGK, right tube, the original strain *L. acidophilus* GK (A). Micrograph of the domesticated strain (B) and the original strain (C).

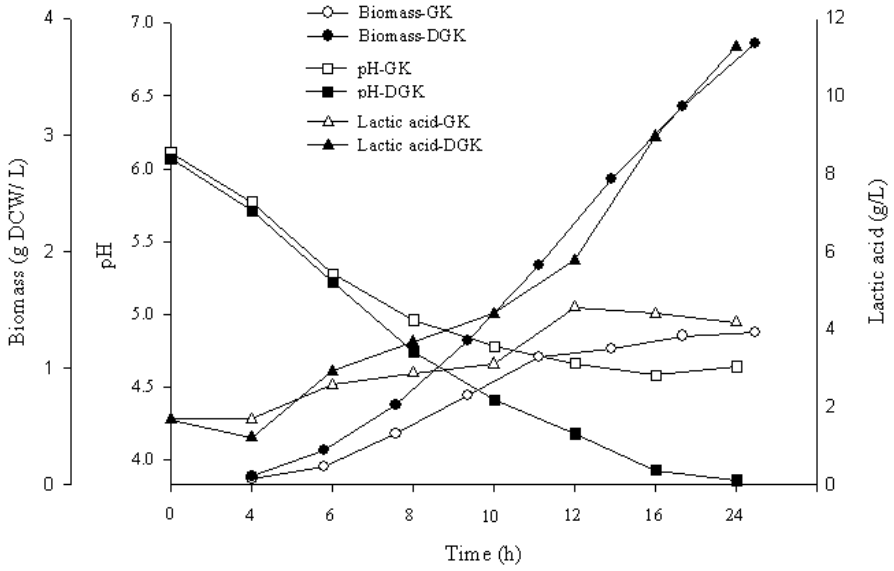


**Figure 2.** Cell density, pH, and lactate concentration of fermented culture by the domesticated strain *L. acidophilus* DGK or the original strain *L. acidophilus* GK in Hinton flasks with shaking (150 rpm). Fermentation conditions: 1% inoculum, 37 ℃, 24 h, 50 mL medium in 500 mL Hinton flask with shaking at 150 rpm. GK: the original strain *L. acidophilus* GK; DGK: the domesticated strain *L. acidophilus* DGK.

strain (2.60 g DCW/L) in MRSC medium under static conditions. These data are consistent with the fact that higher numbers of viable cells, based on the dried weight of cells, were observed in the domesticated strain com-pared to the original strain. These results may prove beneficial for industry as price of commercial products is based on the viable cell number per unit of weight.

Obvious differences in biomass, lactic acid concentration and pH value were observed during fermentation of the domesticated and original strains in an aerobic environment with agitation at 150 rpm (Figure 2). Biomass produced by the domesticated strain (3.79 g DCW/L) was significantly

**Table 1.** Analysis of the ability to metabolize partial carbohydrates from the API 50 CHL test of the domesticated strain *L. acidophilus* DGK and the original strain *L. acidophilus* GK*

| Biochemical test | 24 h | | 48 h | |
|---|---|---|---|---|
| | GK† | DGK | GK | DGK |
| Glycerol | - | - | + | + |
| D-sorbitol | + | - | + | + |
| D-sucrose | + | - | + | + |
| D-raffinose | + | - | + | - |
| Potassium gluconate | - | + | + | + |

*Fermentation conditions: 37°C, 24 or 48 h. †GK, the original strain *L. acidophilus* GK; DGK, the domesticated strain *L. acidophilus* DGK.

higher than that of the original strain (1.31 g DCW/L) during the 24 h fermentation. In addition, a higher concentration of lactic acid (11.28 g/L) was obtained in the domesticated strain and was responsible for the lower pH value that was observed in this strain. More efficient glucose metabolism by the domesticated cells increased biomass, and lactic acid was the major metabolite.

Characteristics of the bacteria before and after domestication were examined. Gram-positive, rod-shape and catalase-negative results were found in both the domesticated cells and the original cells. Resistance to acid and bile salt was also similar (data not shown). Further identification of the strains was performed using the API 50 CHL test kit. The results verified that both strains were *L. acidophilus*. From the distinct results of the biochemical tests by the API 50 CHL kit (Table 1), sorbitol, sucrose and raffinose could not be well utilized by the domesticated strain in a 24 h culture; however, only D-raffinose was undigested in a 48 h culture. The inability to metabolize D-raffinose by the domesticated strain *L. acidophilus* DGK might induce cells to utilize glucose more efficiency and lead to more biomass and lactic acid production. For further confirmation of their similarity, two primers, LB308F and LB806RM, were used to amplify the heat shock protein (hsp) gene sequence. The comparison of the *hsp60* gene sequences of 499 bp between these two strains showed that 99.2% of the DNA sequence was the same (Figure 3). This result further verifies that these two strains are the same species by instinct. The comparative characterization of the domesticated strain of *L. acidophilus* with the original strain has allowed the identification of the phenotypic changes derived from aerotolerance acquisition relevant to probiotic applications.

## Effects of carbon sources on the growth of the domesticated strain

Carbon sources are the most important medium components for cell growth next to basic nutrients, such as amino acids, vitamins or trace elements. In the preliminary medium test, medium composed of glucose 10 g/L, yeast extract 10 g/L, soy peptone (Scharlau) 10 g/L, sodium acetate 5 g/L, ammonium citrate 2 g/L, $MgSO_4.7H_2O$ 0.2 g/L, $MnSO_4.4H_2O$ 0.05 g/L and corn steep liquor 10 mL/L was used to study biomass production under different carbon source replacements (glucose was replaced). The results shown in Table 2 indicate that biomass production of the domesticated strain was significantly higher than that of the original strain. Cell densities of the original strain (2.60 g DCW/L) or the domesticated strain (4.81 g DCW/L) in MRSC medium were higher than those in media substituted with different carbon sources. Before adaptation, maltose and galactose used as the carbon sources allowed greater biomass accumulation (2.02 g and 1.97 DCW/L, respectively) than the other carbon sources. However, after the adaptation, glucose as the carbon source led to the highest biomass production (4.44 g DCW/L), while sucrose, sorbitol and potassium gluconate as carbon sources produced less biomass. Glucose and lactose exhibited higher amounts of biomass production in the domesticated strain. Conversely, the domesticated stain with raffinose or maltose as the carbon source had reduced biomass production. The lower biomass production using raffinose as the carbon source was consistent with the result of the API test, which showed that the domesticated strain cannot metabolize raffinose. We observed that higher biomass correlated to a reduced pH.

Much lower biomass production was obtained when the original strain was incubated while shaking at 150 rpm at 37°C for 24 h (Table 3). The concentration of the cell density in the domesticated strain *L. acidophilus* DGK was significantly increased when cultivating while shaking, even with the different carbon sources, such as glucose, galactose, mannitol and lactose. These results indicate that the domesticated strain had been adapted to aerobic environments and exhibited higher efficiency of metabolism of the carbon sources with the exception of raffinose; although, biomass production was greater when the cultures were cultivated statically versus shaking.

Metabolic changes of carbohydrates have been detected in *Bifidobacterium* strains that have acquired bile tolerance (Sanchez et al., 2005). The aerotolerant derivative, *L. acidophilus* DGK, might display higher fermentative

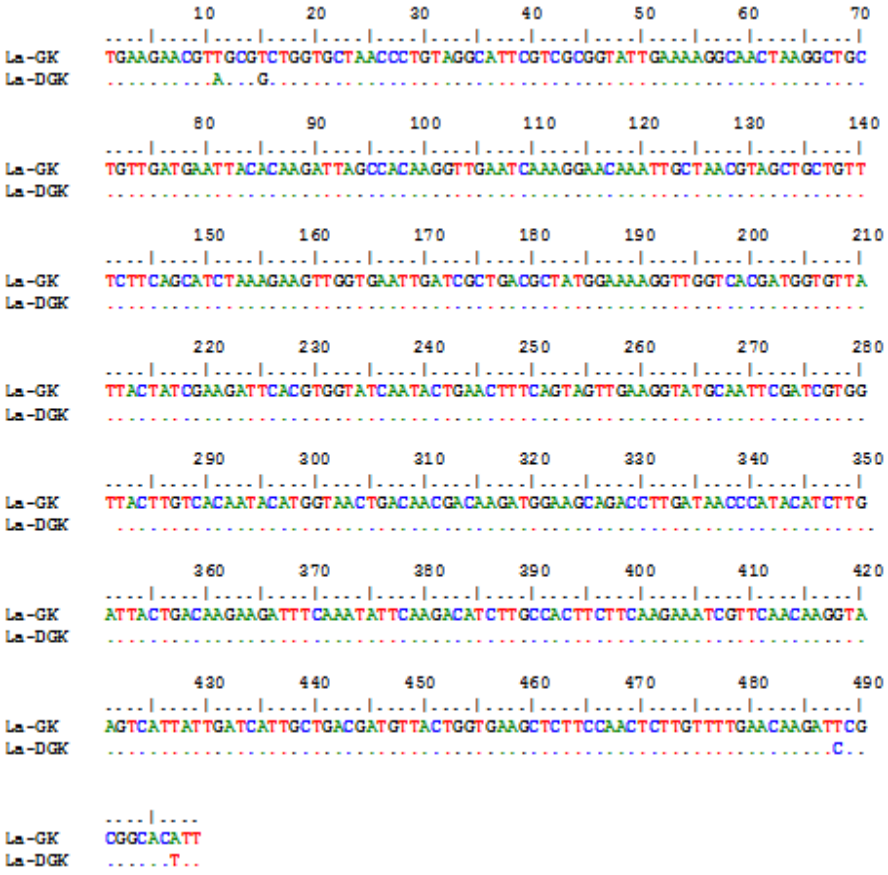


**Figure 3.** Partial DNA sequence of the hsp60 gene from the original strain *L. acidophilus* GK and the domesticated strain *L. acidophilus* DGK.

**Table 2.** Effects of carbon sources on biomass production of the original strain *L. acidophilus* GK and the domesticated strain *L. acidophilus* DGK grown statically

| Medium (control group) | Original strain *L. acidophilus* GK | | Domesticated strain *L. acidophilus* DGK | |
|---|---|---|---|---|
| | Biomass (g DCW/L) | pH | Biomass (g DCW/L) | pH |
| MRSC | 2.60 ± 0.04$^{a\dagger}$ | 3.83 ± 0.01$^{f}$ | 4.81 ± 0.04$^{a}$ | 3.75 ± 0.01$^{i}$ |
| **Carbon source*** | | | | |
| Glucose | 1.30 ± 0.05$^{e}$ | 4.15 ± 0.01$^{c}$ | 4.44 ± 0.14$^{b}$ | 3.80 ± 0.01$^{h}$ |
| Fructose | 1.48 ± 0.02$^{d}$ | 4.15 ± 0.01$^{c}$ | 2.93 ± 0.06$^{e}$ | 3.99 ± 0.01$^{f}$ |
| Galactose | 1.97 ± 0.05$^{b}$ | 4.12 ± 0.01$^{c}$ | 3.74 ± 0.23$^{d}$ | 4.00 ± 0.01$^{f}$ |
| D-Mannitol | 1.25 ± 0.04$^{e}$ | 5.00 ± 0.01$^{b}$ | 2.66 ± 0.09$^{f}$ | 4.60 ± 0.01$^{e}$ |
| Lactose | 1.82 ± 0.05$^{c}$ | 4.07 ± 0.02$^{d}$ | 4.09 ± 0.05$^{c}$ | 3.92 ± 0.01$^{g}$ |
| Sucrose | 0.80 ± 0.13$^{g}$ | 5.22 ± 0.02$^{a}$ | 1.13 ± 0.03$^{i}$ | 5.34 ± 0.01$^{a}$ |
| D-Raffinose | 1.71 ± 0.17$^{c}$ | 4.01 ± 0.01$^{e}$ | 1.13 ± 0.02$^{i}$ | 5.31 ± 0.02$^{a}$ |
| D-Sorbitol | 0.96 ± 0.04$^{f}$ | 5.20 ± 0.01$^{a}$ | 1.25 ± 0.02$^{h}$ | 5.06 ± 0.01$^{c}$ |
| Potassium gluconate | 0.88 ± 0.01$^{f}$ | 5.26 ± 0.01$^{a}$ | 1.17 ± 0.04$^{i}$ | 5.30 ± 0.01$^{a}$ |
| Glycerol | 1.30 ± 0.05$^{e}$ | 4.06 ± 0.02$^{d}$ | 1.30 ± 0.07$^{h}$ | 5.11 ± 0.01$^{b}$ |
| Maltose | 2.02 ± 0.09$^{b}$ | 4.08 ± 0.01$^{d}$ | 1.58 ± 0.02$^{g}$ | 4.90 ± 0.01$^{d}$ |

*Composition of the basal medium: Yeast extract, 10 g/L; Soy peptone, 10 g/L; Sodium acetate, 5 g/L; Ammonium citrate, 2 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; $MnSO_4 \cdot 4H_2O$, 0.05 g/L; Corn steep liquor 10 ml $l^{-1}$. Fermentation conditions: 1% inoculum, 37°C, 24 h, at 10 mL medium/50 mL Hinton flask without shaking. Each value represents the mean ± SD from the experiment conducted in triplicate. $^{\dagger}$Data in the same column with different letters are significantly different at $p<0.05$.

**Table 3.** Effects of carbon sources on the biomass of the original strain *L. acidophilus* GK and the domesticated strain *L. acidophilus* DGK grown while shaking at 150 rpm

| Medium (control group) | Original strain *L. acidophilus* GK | | Domesticated strain *L. acidophilus* DGK | |
|---|---|---|---|---|
| | Biomass (g DCW/L) | pH | Biomass (g DCW/L) | pH |
| MRSC | 1.06 ± 0.04$^{a\dagger}$ | 4.85 ± 0.02$^{g}$ | 4.54 ± 0.07$^{a}$ | 3.96 ± 0.04$^{g}$ |
| **Carbon source*** | | | | |
| Glucose | 0.89 ± 0.03$^{c}$ | 5.12 ± 0.01$^{c}$ | 3.85 ± 0.12$^{c}$ | 4.06 ± 0.02$^{f}$ |
| Fructose | 0.97 ± 0.12$^{b}$ | 5.12 ± 0.02$^{c}$ | 2.06 ± 0.03$^{d}$ | 4.23 ± 0.01$^{e}$ |
| Galactose | 0.66 ± 0.01$^{e}$ | 5.41 ± 0.02$^{a}$ | 4.26 ± 0.29$^{b}$ | 4.06 ± 0.01$^{e}$ |
| D-Mannitol | 0.78 ± 0.03$^{d}$ | 5.35 ± 0.17$^{b}$ | 3.81 ± 0.05$^{c}$ | 3.89 ± 0.12$^{h}$ |
| Lactose | 0.98 ± 0.03$^{b}$ | 5.10 ± 0.01$^{cd}$ | 3.93 ± 0.28$^{c}$ | 4.06 ± 0.01$^{f}$ |
| Sucrose | 0.97 ± 0.01$^{b}$ | 5.03 ± 0.03$^{e}$ | 0.89 ± 0.02$^{g}$ | 5.49 ± 0.01$^{c}$ |
| D-Raffinose | 0.93 ± 0.01$^{b}$ | 5.09 ± 0.03$^{d}$ | 0.82 ± 0.02$^{g}$ | 5.57 ± 0.01$^{b}$ |
| D-Sorbitol | 0.77 ± 0.02$^{d}$ | 5.34 ± 0.01$^{b}$ | 0.97 ± 0.03$^{gf}$ | 5.47 ± 0.01$^{c}$ |
| Potassium gluconate | 0.74 ± 0.01$^{d}$ | 5.42 ± 0.02$^{a}$ | 1.15 ± 0.03$^{ef}$ | 5.85 ± 0.02$^{a}$ |
| Glycerol | 0.75 ± 0.02$^{d}$ | 5.35 ± 0.02$^{b}$ | 0.80 ± 0.03$^{g}$ | 5.49 ± 0.01$^{c}$ |
| Maltose | 0.91 ± 0.02$^{bc}$ | 4.90 ± 0.01$^{f}$ | 1.29 ± 0.10$^{e}$ | 5.34 ± 0.01$^{d}$ |

*Composition of the basal medium is the same as Table 2. $^{\dagger}$Data in the same column with different letters are significantly different at $p<0.05$.

ability to carbohydrates and increased enzymatic activities, such as glucosidase, which could favor carbohydrates metabolism. The derived strain might improve carbohydrate hydrolysis activity and be more suitable for application in the production process of probiotic cells.

### Effects of nitrogen sources on the growth of the domesticated strain

A basal medium (PM) consisting of glucose (20 g/L), yeast extract (10 g/L, sodium acetate (5 g/L) and $MnSO_4 \cdot 4H_2O$ (0.05 g/L) was used to study the supplementation of different nitrogen sources (10 g/L) under static or shaking fermentation (Tables 4 and 5). The results presented in Table 4 show that, in the original strain under static conditions, higher biomass was obtained when corn steep liquor (CSL) was the nitrogen source versus other nitrogen sources. CSL is an important nitrogen alternative to peptone or yeast extract. CSL contains amino acids and vitamins that may be beneficial for the growth of LAB. Biomass production was significantly enhanced in the domesticated strain *L. acidophilus* DGK compared to the parental strain. Among the nitrogen sources examined in the present study, beef extract, peptone, ammonium citrate and monosodium glutamate (MSG) produced a higher amount of cells in the static conditions. Cheaper organic nitrogen sources, such as MSG and CSL, and inorganic nitrogen sources, such as ammonium citrate, can be used as nitrogen alternatives to reduce production costs.

Table 5 shows the effect of different nitrogen sources on biomass production under shaking conditions. When comparing the biomass produced by the domesticated strain from Table 4 and Table 5, we found that higher biomass was obtained in shaking conditions, compared to static conditions, when beef extract, malt extract, soy peptone, yeast peptone and tryptone were used as the nitrogen sources. In contrast, ammonium citrate as the nitrogen source produced more biomass in the static situation. Thus, the improvement of biomass production depended on both the nitrogen source and the cultivation environment. Medium with urea as the nitrogen source in both the static and shaking conditions produced the lowest biomass (0.81 g DCW/L), indicating that this inorganic compound was not suitable for the growth of LAB.

## DISCUSSION

In the present study, it was shown that characteristics of LAB could be modified through an adaptation strategy by successively cultivating strains under specific conditions and screening the stable strains. Some papers have described the modification of strains by adapting them in extreme environments and have revealed difficulties for maintaining their stabilities. In this study, we isolated the stable strain *L. acidophilus* DGK not only by successive subculture for 14 days in aerobic conditions but also through population analysis of over 400 colonies. The aerotolerant strain had increased biomass production in aerobic environments and possessed an improved efficiency of nutrient utilization as well as production of lactic acid. This modified strain might be more adaptable to the stirring process of pH control and the feeding strategy used during fed-batch fermentation. The identification of the domesticated strain by API 50 CHL and hsp60 gene sequence analysis confirmed that the modified strain was derived from the starting strain and had improved growth characteristics and nutrition

**Table 4.** Effects of nitrogen sources on the biomass production of the original strain *L. acidophilus* GK and the domesticated strain *L. acidophilus* DGK grown statically.

| Medium (control group) | Original strain *L. acidophilus* GK | | Domesticated strain *L. acidophilus* DGK | |
|---|---|---|---|---|
| | Biomass (g DCW/L) | pH | Biomass (g DCW/L) | pH |
| MRSC | 2.56 ± 0.03$^{a\dagger}$ | 3.76 ± 0.01$^{k}$ | 04.59 ± 0.35$^{a}$ | 3.67 ± 0.02$^{e}$ |
| PM* | 0.06 ± 0.01$^{i}$ | 5.55 ± 0.01$^{f}$ | 02.54 ± 0.05$^{g}$ | 3.68 ± 0.01$^{ih}$ |
| **Nitrogen source to PM*** | | | | |
| Beef extract | 0.11 ± 0.01$^{d}$ | 5.46 ± 0.02$^{f}$ | 2.98 ± 0.11$^{ef}$ | 3.74 ± 0.01$^{ef}$ |
| Malt extract | 0.10 ± 0.01$^{de}$ | 5.39 ± 0.02$^{g}$ | 1.18 ± 0.04$^{c}$ | 3.68 ± 0.02$^{h}$ |
| Corn steep liquor | 1.04 ± 0.02$^{b}$ | 4.19 ± 0.04$^{j}$ | 3.32 ± 0.23$^{c}$ | 3.90 ± 0.01$^{h}$ |
| Peptone | 0.09 ± 0.01$^{efg}$ | 5.54 ± 0.03$^{d}$ | 3.31 ± 0.07$^{c}$ | 3.71 ± 0.01$^{fg}$ |
| Soy peptone | 0.09 ± 0.01$^{ef}$ | 5.40 ± 0.02$^{g}$ | 1.42 ± 0.05$^{j}$ | 3.92 ± 0.01$^{c}$ |
| Yeast peptone | 0.10 ± 0.01$^{de}$ | 5.50 ± 0.02$^{e}$ | 1.46 ± 0.06$^{j}$ | 3.71 ± 0.01$^{g}$ |
| Tryptone | 0.10 ± 0.01$^{de}$ | 5.38 ± 0.01$^{g}$ | 1.48 ± 0.03$^{j}$ | 3.68 ± 0.01$^{h}$ |
| Ammonium citrate | 0.07 ± 0.01$^{hi}$ | 5.80 ± 0.01$^{b}$ | 3.86 ± 0.03$^{b}$ | 3.99 ± 0.01$^{b}$ |
| Ammonium chloride | 0.09 ± 0.01$^{ef}$ | 5.30 ± 0.01$^{ih}$ | 2.19 ± 0.02$^{h}$ | 3.56 ± 0.01$^{h}$ |
| Ammonium nitrate | 0.08 ± 0.01$^{hfg}$ | 5.54 ± 0.02$^{ih}$ | 1.98 ± 0.03$^{i}$ | 3.66 ± 0.01$^{ih}$ |
| Ammonium sulfate | 0.08 ± 0.01$^{hfe}$ | 5.34 ± 0.01$^{h}$ | 2.29 ± 0.03$^{g}$ | 3.61 ± 0.01$^{j}$ |
| Ammonium phosphate | 0.13 ± 0.01$^{c}$ | 5.72 ± 0.01$^{c}$ | 2.72 ± 0.10$^{h}$ | 3.71 ± 0.01$^{g}$ |
| Urea | 0.04 ± 0.01$^{j}$ | 5.84 ± 0.04$^{a}$ | 0.81 ± 0.08$^{k}$ | 5.06 ± 0.04$^{a}$ |
| MSG | 0.07 ± 0.01$^{hig}$ | 5.56 ± 0.01$^{d}$ | 13.71 ± 0.05$^{d}$ | 3.82 ± 0.01$^{d}$ |
| $H_2O$ | 0.07 ± 0.01$^{hi}$ | 5.53 ± 0.04$^{de}$ | 1.48 ± 0.03$^{g}$ | 3.64 ± 0.02$^{i}$ |

*Composition of basal medium (PM): Glucose, 20 g/L; Yeast extract, 10 g/L; Sodium acetate, 5 g/L; $MnSO_4 \cdot 4H_2O$, 0.05 g/L; nitrogen source added: 10 g/L. †Data in the same column with different letters are significantly different at $p<0.05$.

**Table 5.** Effects of nitrogen sources on biomass production of the original strain *L. acidophilus* GK and the domesticated strain *L. acidophilus* DGK grown while shaking at 150 rpm

| Medium (control group) | Original strain *L. acidophilus* GK | | Domesticated strain *L. acidophilus* DGK | |
|---|---|---|---|---|
| | Biomass (g DCW/L) | pH | Biomass (g DCW/L) | pH |
| MRSC | 0.97 ± 0.16$^{a\dagger}$ | 4.06 ± 0.01$^{l}$ | 4.79 ± 0.11$^{a}$ | 3.90 ± 0.01$^{c}$ |
| PM* | 0.07 ± 0.01$^{e}$ | 5.55 ± 0.01$^{de}$ | 3.10 ± 0.02$^{e}$ | 3.69 ± 0.01$^{h}$ |
| **Nitrogen source to PM*** | | | | |
| Beef extract | 0.11 ± 0.01$^{b}$ | 5.53 ± 0.01$^{de}$ | 3.62 ± 0.04$^{b}$ | 3.74 ± 0.01$^{g}$ |
| Malt extract | 0.11 ± 0.01$^{b}$ | 5.43 ± 0.02$^{fh}$ | 2.88 ± 0.12$^{ef}$ | 3.69 ± 0.01$^{h}$ |
| Corn steep liquor | 0.96 ± 0.06$^{a}$ | 4.85 ± 0.01$^{k}$ | 3.39 ± 0.04$^{c}$ | 3.74 ± 0.01$^{g}$ |
| Peptone | 0.09 ± 0.01$^{cde}$ | 5.61 ± 0.02$^{d}$ | 3.64 ± 0.08$^{b}$ | 3.81 ± 0.01$^{e}$ |
| Soy peptone | 0.09 ± 0.01$^{cde}$ | 5.60 ± 0.13$^{d}$ | 3.62 ± 0.02$^{b}$ | 3.78 ± 0.01$^{f}$ |
| Yeast peptone | 0.11 ± 0.01$^{cd}$ | 5.45 ± 0.02$^{ghi}$ | 3.54 ± 0.11$^{b}$ | 3.76 ± 0.01$^{fg}$ |
| Tryptone | 0.10 ± 0.01$^{d}$ | 5.47 ± 0.02$^{fgh}$ | 3.57 ± 0.05$^{b}$ | 3.74 ± 0.01$^{g}$ |
| Ammonium citrate | 0.07 ± 0.01$^{e}$ | 5.83 ± 0.01$^{b}$ | 2.02 ± 0.10$^{i}$ | 4.16 ± 0.01$^{b}$ |
| Ammonium chloride | 0.08 ± 0.01$^{cde}$ | 5.42 ± 0.01$^{hi}$ | 2.63 ± 0.10$^{h}$ | 3.59 ± 0.01$^{k}$ |
| Ammonium nitrate | 0.08 ± 0.01$^{cde}$ | 5.40 ± 0.01$^{ij}$ | 1.95 ± 0.04$^{i}$ | 3.77 ± 0.04$^{f}$ |
| Ammonium sulfate | 0.07 ± 0.01$^{e}$ | 5.34 ± 0.05$^{j}$ | 2.75 ± 0.02$^{g}$ | 3.63 ± 0.01$^{j}$ |
| Ammonium phosphate | 0.13 ± 0.01$^{b}$ | 5.73 ± 0.01$^{c}$ | 2.96 ± 0.05$^{e}$ | 3.84 ± 0.01$^{d}$ |
| Urea | 0.04 ± 0.01$^{f}$ | 5.93 ± 0.03$^{a}$ | 0.73 ± 0.07$^{j}$ | 5.14 ± 0.01$^{a}$ |
| MSG | 0.08 ± 0.01$^{de}$ | 5.55 ± 0.02D$^{e}$ | 3.27 ± 0.09$^{d}$ | 3.93 ± 0.01$^{c}$ |
| $H_2O$ | 0.08 ± 0.01$^{e}$ | 5.50 ± 0.02$^{efg}$ | 2.81 ± 0.03$^{fg}$ | 3.65 ± 0.01$^{i}$ |

*Composition of basal medium (PM) is the same as Table 4. †Data in the same column with different letters are significantly different at $p<0.05$.

utilization. This change may be due to gene modification during the adaptation process. Stability of biomass production by this modified strain was verified by repeated cultivation and analysis. The process of domestication and population analysis for screening for the enhancement of desired characteristics might be applicable to other probiotics in the industrial production of LAB starter or chemical-producing strains.

An aerotolerant probiotic strain, *L. acidophilus* DGK, displaying improved biomass production and nutrient utilization might be due to gene modification. It would be particularly worthwhile to further study the proteins induced in the DGK strain during aerobic stress, to identify those proteins that are important for increased tolerance to oxygen stress. It is possible that this adaptation could enhance the biomass production during industrial fermentation and might also lengthen the viability of this probiotic strain during storage at room temperature. The application of prolonged exposures to aerobic conditions has been particularly useful to generate stable and highly aerotolerant *L. acidophilus* strains from naturally aerobically sensitive strains. This treatment also intro-duced phenotypic changes that could lead to strains with improved biological properties, although each derivative strain requires further individual evaluation.

The ability to metabolize raffinose was inhibited in the domesticated strain, as was shown by the API assay and carbon source replacement experiments. Glucose, galactose and lactose were efficiently utilized by the domesticated strain in both the static and shaking conditions.

The cell growth of the modified strain was significantly improved by the addition of various nitrogen sources either under static or shaking conditions. The effect of these additions was more significant in the shaking conditions than in the static conditions. By comparing the efficiency of carbon and nitrogen source replacement by the domesticated strain, we conclude that both the fermentation environments and the carbon and nitrogen sources highly impact biomass production. The meta-bolism and utilization of nutrients by the modified strain varied not only based on the growth environment but also on the varieties of nutrients available to the strain.

## Conflict of interests

The authors have not declared any conflict of interest.

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*Full Length Research Paper*

# Induction and characterization of pathogenesis-related proteins in roots of cocoyam (*Xanthosoma sagittifolium* [L] Schott) infected with *Pythium myriotylum*

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**Although *Pythium myriotylum* is a very destructive root pathogen of cocoyam, the host defense response in this plant-pathogen interaction has not been fully studied. Four cocoyam germplasm accessions were inoculated with *P. myriotylum*, and their induced defense responses were characterized. The induction and spatio-temporal accumulation of chitinase and β-1,3-glucanase were determined by enzymatic activity assays of crude root extracts from inoculated and non-inoculated (control) plants, sampled at 0, 2, 4, 6 and 8 days post inoculation (dpi). Furthermore, induced proteins were extracted from roots of inoculated and control tolerant (RO1054 and RO3015) and susceptible (RO2063) accessions at 8 dpi, and characterized by isoelectric focusing (IEF), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses. Chitinase and β-1,3-glucanase were consistently produced in high amounts in the roots of the tolerant accession RO1054, 8 days after inoculation. SDS-PAGE and immunoblotting showed that induced chitinases (37, 35 and 33 kDa) in the tolerant cocoyams were immunologically related to PR-3a purified from barley leaves inoculated with *Erysiphe graminis* f. sp. *hordei*, and induced osmotins (42-45 kDa) were immunologically related to osmotins purified from cultured NaCl-adapted tobacco protoplasts. These results suggest that tolerance in cocoyam infected with *P. myriotylum* may be associated in part with the production of pathogenesis-related (PR) proteins including one hydrolytic enzyme of known antifungal activity (PR-3).**

**Key words:** β-1,3-glucanase, chitinase, cocoyam, PR protein, *Pythium myriotylum*, osmotin.

## INTRODUCTION

Cocoyam (*Xanthosoma sagittifolium* (L) Schott), in the family Araceae, is a staple food for millions of inhabitants in the humid tropics and subtropics where tubers and leaves are consumed as a source of carbohydrates, minerals and vitamins. Nigeria is the world's largest producer where production increased from 3.89 million

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**Table 1.** Morphological characteristics and putative levels of resistance of cocoyam accessions used for the study

| Accession[a] | Color of petiole/petiole base | Tuber flesh color | Reaction to *P. myriotylum*[b] |
|---|---|---|---|
| "Local White" | Green/white | White | Susceptible |
| RO1054 | Green/green | White | Tolerant |
| RO2063 | Purple/pink | Pink | Susceptible |
| RO3015 | Purplish/white | Yellowish/white | Fairly resistant |

[a]Accessions RO1054, RO2063 and RO3015 were selections of the Cocoyam Breeding Program, Institute of Agricultural Research for Development (IRAD), Ekona, Cameroon. [b]Data obtained from Jay P. Johnson Biotechnology Laboratory, ROTREP Cocoyam Breeding Program, Institute of Agricultural Research for Development (IRAD), Ekona, Cameroon.

tons in 2000 to 5.068 tons in 2007 (Olagunju and Adesiji, 2011). The cocoyam root rot disease caused by *P. myriotylum* Drechs is the most damaging disease of cocoyam. As part of an ongoing research effort to improve cocoyam, notably in the development of cultivars that are resistant/tolerant to the root rot disease, the causal pathogen has been well characterized (Pacumbaba et al., 1992; Pacumbaba, 1996; Tambong et al., 1999). Although resistance or tolerance to the cocoyam root rot disease has been observed in some cocoyam types, however, the mechanism of tolerance/ resistance is not known.

Plants possess various defense mechanisms that enable them to survive under biotic or abiotic stress. Among several defense tactics, induced defense mechanisms against plant pathogens which involve the hypersen-sitive response, reactive oxygen species, and pathogenesis-related (PR) proteins have received considerable research attention in many crop species (Hammond-Kosack and Jones, 1996; Rahimi et al., 1996; Reiss and Bryngelsson, 1996; Van Loon, 1999). Pathogenesis-related (PR) proteins are a group of plant proteins some of which are normally present in low amounts in the non stressed plant, but whose production becomes massively increased and new ones induced, when plants are subjected to biotic or abiotic stresses. Many of these proteins associated with host defense have been characterized as chitinases, ß-1,3-glucanases, peroxidases and thaumatin-like proteins (TLP). Among the PR proteins, chitinases, glucanases and osmotins expressed in plants attacked by fungi are thought to limit fungal growth. This antifungal biological function has been demonstrated *in vitro* against several fungi (Anfoka and Buchenauer, 1997; Ji and Kie, 1996; Lawrence et al., 1996; Yun et al., 1996; Zhang et al., 1996). The hydrolases, chitinase and glucanase which degrade the ß-1,4-linkage between N-acetylglucosamine residues of chitin and glucans in fungal cell walls, and thaumatin-like proteins are also known to be induced in plants by adverse environmental factors (Bowles, 1990; Cruz-Ortega and Ownby, 1993; Hincha et al., 1997; Yalpani et al., 1994).

Another indirect role of ß-1,3-glucanases and chitinases is that they act as elicitors of defense reactions, that is they release oligosaccharides from cell walls of both fungi and plants that in turn activate the accumulation of phytoalexins, extensins, proteinase inhibitors and lignin in the attacked host plant (Ham et al., 1991). PR proteins occur in many plants where they also play a developmental role (Leubner-Metzger and Meins, 1999).

Due to the involvement of PR proteins in host resistance, genes encoding such PR proteins could be exploited to engineer resistance in crops (Sharma et al., 1993). The root rot problem in cocoyam could be solved if genes conditioning resistance against *Pythium myriotylum* were identified and either bred in or cloned and genetically engineered into cocoyam. A logical first step would be to understand the host resistance response that is deployed during pathogen infection or attempted infection. Also, identified genes may be targeted for over-expression to develop resistant cultivars. Broglie et al. (1991) found that transgenic plants over-expressing a basic bean chitinase exhibited increased resistance against *Rhizoctonia solani*.

The objective of this study was to determine and characterize host-specific defense related proteins induced in cocoyam, their serological relationship to known PR proteins and if there is temporal accumulation of these PR proteins in response to *P. myriotylum* infection.

## MATERIALS AND METHODS

### Plant inoculation and protein extraction for enzymatic activity assays

Five plants from each of the cocoyam breeding accessions RO3015, RO2063, RO1054 and "Local White" (Table 1) were inoculated at the roots with a suspension of $1 \times 10^4$ mycelial fragments/ml or zoospores of *P. myriotylum* or distilled water in a split root system. *P. myriotylum* used for inoculation was a gift from Dr. Tambong. A hyphal tip was grown on lima bean sucrose agar (LBSA, pH 7.0) at 31°C in the dark for 5 days prior to being used for inoculum preparation. The inoculum was derived by macerating 5-day old flocculent mycelia cultures with distilled deionized water in a Waring blender. The final inoculum concentration was adjusted to $1 \times 10^4$ mycelial fragments per ml. The split root system was set up such that about 8-10 cocoyam roots in the potted soilless medium were carefully extended through the holes at bottom of the pot into

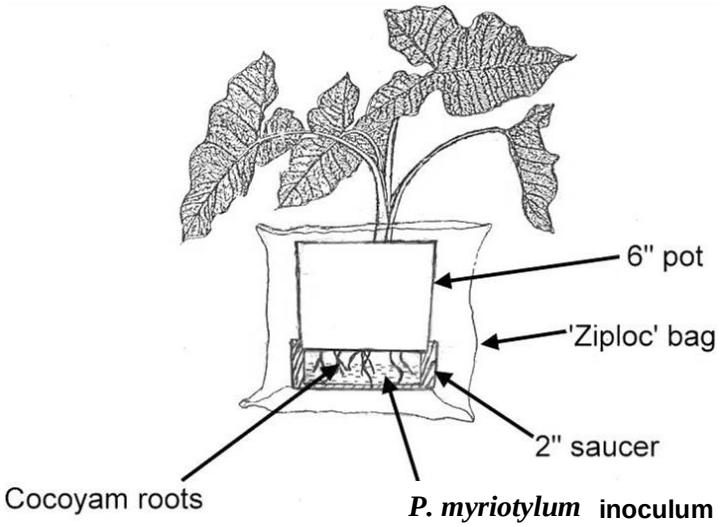



**Figure 1**. Schematic of the split root method used in inoculating cocoyam plants to study induced defense responses. The inoculated roots were maintained in the pathogen inoculum until time of sample collection.

a Pm inoculum containing saucer placed outside the bottom of the pot, while the rest of the roots remained undisturbed in the potted soil (Figure 1). This set up was used in order to exclude pathogen proteins in the analysis. During sample collection, only non-inoculated root samples from inoculated and control plants, were collected at 0, 2, 4, 6 and 8 days post inoculation for protein extraction and further analysis by specific enzymatic activity assays.

For SDS-PAGE, IEF and Immunobloting, non-inoculated root samples from control and inoculated RO1054, RO2063 and RO3015 plants, were collected 8 days after inoculation for protein extraction and analysis. Since there was no resistant cocoyam, these accessions were selected to represent tolerant (partially resistant) and susceptible cocoyams only. The eighth day after inoculation was chosen for sample collection because it represented the time point where the maximum enzyme activity for inoculated sample plants of these accessions was recorded in preliminary experiments. Root samples were stored at -70°C for 48 h followed by crude protein extraction. The frozen roots were ground (10:1; w/v) in extraction buffer (pH 6.8), containing 0.1n M Tris-HCl, 1% (w/v) polyvinylpyrollidone (PVP:40) and 1% (v/v) 2-mercaptoethanol, with a mortar and pestle at 4°C. Samples were sieved through autoclaved cheesecloth and centrifuged at 15,000 rpm for 20 min at 4°C.

**Concentration and partial purification of samples**

After centrifugation, the supernatant was mixed (1:5 v/v) with ice-cold acetone, incubated at -20°C for 2 min to precipitate proteins (Bollag et al., 1996) or was precipitated with ammonium sulfate at 85% saturation (Bollag et al., 1996). The precipitate was centrifuged at 10,000 rpm for 10 min at 4°C. The protein pellet was re-dissolved in 0.1 M Tris-HCl, pH 6.8, containing 5% (v/v) 2-mercaptoethanol and 20% (v/v) glycerol. Aliquots of 100 µl were placed in 1.5 ml microfuge tubes and stored at -70°C until used. Protein samples were desalted and further purified using Micro Bio-Spin® 6 chromatography columns (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The protein concentration of the purified sample was then determined.

**Determination of protein concentration**

The protein concentration in all samples was measured according to the method of Bradford, using a protein kit (Bio-Rad, Hercules, CA) and samples were used for electrophoresis without further purification.

**Assays for enzymatic activity**

***Chitinase assay***

The substrate used for this assay was carboxymethyl chitin remazol brilliant violet (CM-chitin RBV, 2 mg/ml; Lowe Biochemica, Germany). The reaction mixture in a 1.5 ml microfuge tube consisted of 100 µl of substrate, 200 µl of 0.1 M sodium acetate buffer, pH 5.0 and 100 µl crude protein extract from cocoyam roots. The mixtures were incubated for 1, 10, 20 and 30 min at 37°C. The reacted samples were centrifuged at 5000 *g* for 10 min and supernatant read at 550 nm. The log phase of the slope of the resultant curve was used to calculate the enzymatic activity, which was expressed as units/µg protein. One unit was equivalent to 1 µg CM-chitin hydrolyzed per minute.

***ß-1,3-glucanase assay***

Carboxymethyl-curdlan remazol brilliant blue (CM-curdlan RBB, 4 mg/ml; Lowe Biochemica, Germany) was used as glucanase substrate. The assay mixture contained 100 µl of substrate, 200 µl of 0.1 M sodium acetate buffer, pH 5.0 and 100 µl crude protein extract. The mixture was incubated at 37°C at 1, 15, 30 and 60 min, then centrifuged at 5000 *g* for 10 min and supernatant read at 595 nm (Microplate Reader Model 550; Bio-Rad, Hercules, CA). Enzymatic activity was determined as described for chitinase. One unit of activity was equal to 1 µg CM-curdlan hydrolyzed per minute.

**SDS-PAGE**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was

carried out on 0.75 mm 18% (30:0.8 acrylamide/bis-acrylamide) slab gels to determine purity of the crude protein extracts, differences between samples, and resolve proteins for western blotting, according to the protocols of Bollag et al. (1996). The resolving gel was prepared by combining acrylamide (18.48%), 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 50 µl 10% ammonium persulfate and 5 µl TEMED. The stacking gel (5%) contained 5.159% total acrylamide, 0.125 M Tris-HCl, pH 6.8, 0.04% SDS, 30 µl of 10% ammonium persulfate and 5 µl TEMED. Mini gels (6 cm x 8 cm x 0.75 mm) were cast in slabs according to the BIO-RAD mini gel procedures (Bio-Rad, Hercules, CA). After gels were cast, protein samples were combined (4:1) with sample buffer (5x) containing 60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol and 0.1% bromophenol blue. The solution was mixed and heated at 95°C for 4 min, centrifuged briefly (1 s) and sample volumes corresponding to approximately 20 µg was loaded in each well. Samples were resolved at 150 V constant voltage for 1.4 h. Proteins were fixed and stained for 8-10 min in Coomassie gel stain solution containing 0.1% Coomassie Blue R-250, 45% methanol and 10% glacial acetic acid. Gels were destained overnight in a solution containing 10% each of methanol, and glacial acetic acid, photographed and image processed using the AlphaImager® computer software. The molecular masses of resolved proteins were estimated by coelectrophoresis of marker proteins (Bio-Rad) of molecular masses ranging from 6.5 to 200 kDa.

#### Isoelectric focusing and detection of chitinase and glucanase in overlay gels

Isoelectric focusing was carried out on 0.4 mm gels using the Bio-Rad Mini IEF Cell (Bio-Rad, Hercules, CA). The gels were prepared by combining 2 ml 24.25% acrylamide+0.75% bis-acrylamide, 0.5 ml water-free glycerol, 500 µl 3/10 ampholyte and 5.5 ml distilled water. After degassing the solution for 5 min, 15 µl ammonium persulfate, 50 µl 0.1 % riboflavin and 3 µl TEMED were added and gels cast according to the equipment manufacturer's protocol. Protein samples were loaded (5 µl, approx. 4.5 µg/well) directly on the gel. Samples were focused for 15 min at 100 V, 15 min at 200 V and 1 h at 450 V. Overlay slab gels (7.5%) were cast according to the protocol of Bollag et al. (1996). They contained either 0.04% CM-Chitin RBV or CM-Curdlan RBB in 0.2 M sodium acetate buffer, pH 5.0.

#### Detection of enzyme activity on overlay gels

After IEF was complete, the focused gels were overlaid with the overlay gels and the gels sandwich incubated under moist conditions at 37°C overnight (12 h). Chitinase was detected by incubating the overlay gel in freshly made 0.01% (w/v) fluorescent brightener 28 (Calcofluor white M2R) in 0.5 M Tris-HCl (pH 8.8) at 26°C for 10 min. The gels were rinsed in distilled deionized water at 26°C for 10-15 min. Chitinase isozymes were visualized as clear zones on a UV light source. Gel pieces representing different bands were cut and incubated in distilled deionized water overnight after which the pH of each piece was determined.

#### Western blotting

SDS-PAGE was carried out using 18% polyacrylamide gels as described earlier. After SDS-PAGE, proteins were transferred onto Immun-Blot® PVDF or nitrocellulose membranes using the Bio-Rad Mini Transblot® electrophoretic transfer cell (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Electroblotting was completed in 1 h at 100 V, constant voltage. After electro-transfer, the membrane was blocked for 1 h with 1% (w/v) BSA in Tris buffered saline (TBS; 20mM Tris, 500mM NaCl, pH 7.4) containing 0.05% Tween 20 (TBS-T) and washed with TBS-T twice for 15 min each. The primary polyclonal antibodies for chitinase (PR-3) and glucanase (PR-2) were gifts from Dr. Tomas Bryngelsson; the antibodies were raised from barley leaves inoculated with the powdery mildew fungus, *Erysiphe graminis* f. sp *hordei* syn. *Blumeria graminis* f. sp *hordei*. Anti-osmotin antibody raised against osmotin purified from NaCl- adapted cultured tobacco cells, a gift from Dr. Meena Narsimhan, Purdue University, USA, was also used. Anti-PR-3 anti-chitinase and anti-PR-2 anti-glucanase antibodies were diluted 1:250, while anti-osmotin was diluted 1:3000, in blocking buffer. The antibodies were incubated in this solution overnight (11 h) at room temperature. The next day, the membranes were washed in two changes of TBS-T each for 15 min and subsequently incubated for 2 h in the secondary antibody diluted 1:1000 (goat anti-rabbit IgG [H+L] alkaline phosphatase conjugate in 10mM Tris, 150mM NaCl, 1mM $MgCl_2$, pH 8.0) (Bio-Rad, Hercules, CA) for chitinase and glucanase, and 1:5000 (rabbit anti-chicken IgG [H+L] alkaline phosphatase conjugate in 10mM Tris-HCl, 250 mM NaCl, pH 8.0) (Jackson ImmunoResearch Laboratories Inc, West Grove, PA) for osmotin, with blocking buffer, followed by two washes with TBS-T as above. The protein bands were revealed by incubating the membranes with constant shaking in 25 ml of alkaline phosphatase substrate containing 250 µl nitroblue tetrazolium (NBT) in aqueous dimethyl formamide [DMF] containing $MgCl_2$ and 250 µl BCIP (5-bromo-4-chloro-3-indoyl phosphate in DMF). Color development was stopped, by rinsing the membranes three times in distilled water.

#### Statistical analysis of data

Enzymatic activity measurements were subjected to the analysis of variance procedure. Treatment means were compared using Tukey HSD test ($p \leq 0.05$). The SAS statistical software was used in all data analyses.

## RESULTS

### Chitinase activity

There was a significant increase in chitinase activity in roots of all inoculated plants, compared to the controls. Constitutive levels of chitinase activity ranged from 10-60 units/µg of protein. Chitinase activity increased up to 4-fold in roots of inoculated RO1054 (tolerant) plants at 8 dpi (Figure 2A). In the susceptible "Local White", chitinase activity also increased significantly (up to 6-fold) in roots of inoculated plants at 6 dpi (Figure 2B), and then sharply declined. The time course of chitinase activity in the tolerant RO3015 showed significant activity in roots of inoculated plants at 4, 6 and 8 dpi (Figure 2C), while in the susceptible RO2063, high and significant chitinase activity was observed at 6 and 8 dpi (6-fold increase) (Figure 2D). These results show there was a systemic increase in chitinase activity, in cocoyam roots resulting from inoculation with *P. myriotylum*.

### ß-1,3-glucanase

ß-1,3-glucanase activity was only significantly induced in

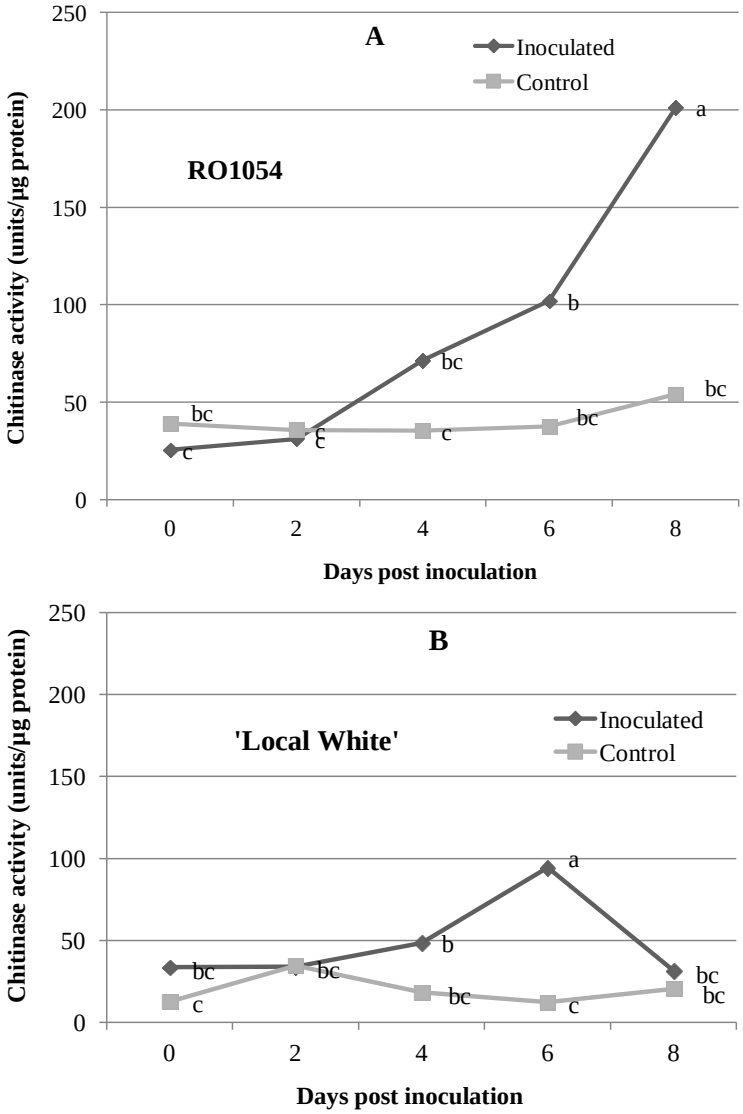



**Figure 2.** Chitinase activity in non inoculated roots of cocoyam accessions RO1054 (**A**), 'Local White' (**B**), RO3015 (**C**) and RO2063 (**D**) infected with *P. myriotylum*.

roots of inoculated tolerant RO1054 at 8 dpi (Figure 3). No significant increase in activity occurred in roots of inoculated RO2063, "Local White" and RO3015 (data not shown). Based on these results, it could be concluded that the induction of ß-1,3-glucanase activity was mainly associated with roots of the tolerant cocoyam RO1054 as roots of inoculated susceptible plants showed no apparent change in ß-1,3-glucanase activity.

## IEF

Two basic chitinase isozymes (pI 8.5 and 8.6) exhibiting activity in overlay gels (visualized as clear zones in UV light), were detected in roots of inoculated RO3015, RO2063 and RO1054 (Figure 4). The isozymes were constitutively present in RO2063 and RO1054, but their activity increased in inoculated plants as shown by the intensity of the bands. Evidently, more of the protein was expressed in RO2063 and RO1054 compared to RO3015. Another isozyme (pI 8.3) was weakly expressed in roots of inoculated RO2063 and RO1054 only. β-1,3-glucanase activity was not detected in overlay gels using the substrate CM-Curdlan RBB.

## SDS-PAGE and Western blot analysis

Polypeptide profiles of protein extracts from roots of inoculated and control RO3015, R02063 and RO1054

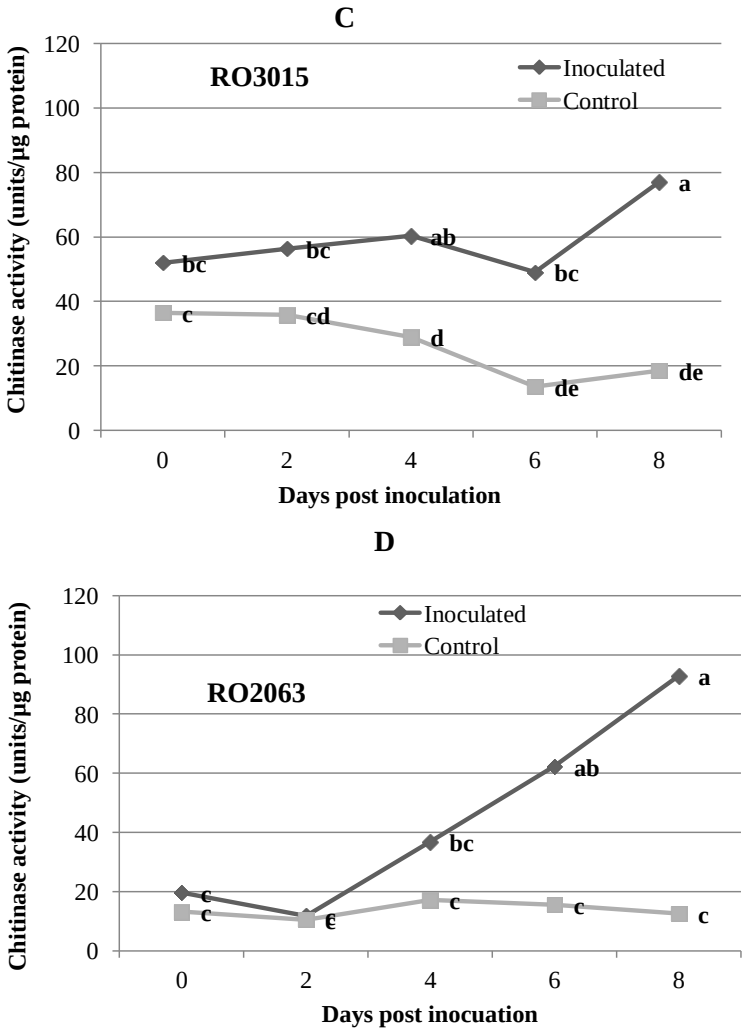



**Figure 2.** Contd.

plants, sampled 8 days post inoculation, showed only one new protein band (26 kDa) in RO3015, one new band (24 kDa) in RO2063, and three bands (24, 31, 37 kDa) in RO1054. Some protein bands however, were not clearly visible in the gel photographs after processing.

Immunoblotting of proteins using anti-PR-3 raised against chitinase induced in barley leaves inoculated with the powdery mildew fungus *Blumeria graminis* f. sp. *hordei*, revealed one protein band with a molecular mass of 35 kDa and detected in both inoculated and control plants of RO1054 and RO3015. In addition, one new chitinase band (37 kDa) was detected in inoculated RO1054 and one band (35-36 kDa) detected in inoculated RO3015 and RO2063 plants (Figure 5). The protein band of 33kDa also detected in inoculated plants of RO1054 was detected only in control plants of RO3015. These chitinase bands are isoforms of the PR-3 protein. In tomato, Joosten et al. (1995), purified four chitinase isoforms with molecular weights 26, 27, 30 and 32 kDa belonging to PR-3a, -3d, -3b and -3c respectively. Lawrence et al. (1996), reported similar findings in tomato. The PR-3 protein isoforms detected in cocoyam did not share identical molecular weight with the published isoforms detected in tomato. Anti-osmotin polyclonal antibodies purified from NaCl-adapted tobacco cells, detected a 42-45 kDa protein band in inoculated RO1054, RO2063 and RO3015. This protein was absent in the controls Yc and Rc but present in Wc (RO1054) (Figure 6). This indicates that the protein maybe constitutively expressed in RO1054, an accession which exhibited significant tolerance to *P*. *myriotylum*.

## DISCUSSION

The main objective of this study was to investigate host defense mechanisms in susceptible and tolerant cocoyams inoculated with *P*. *myriotylum*. We observed

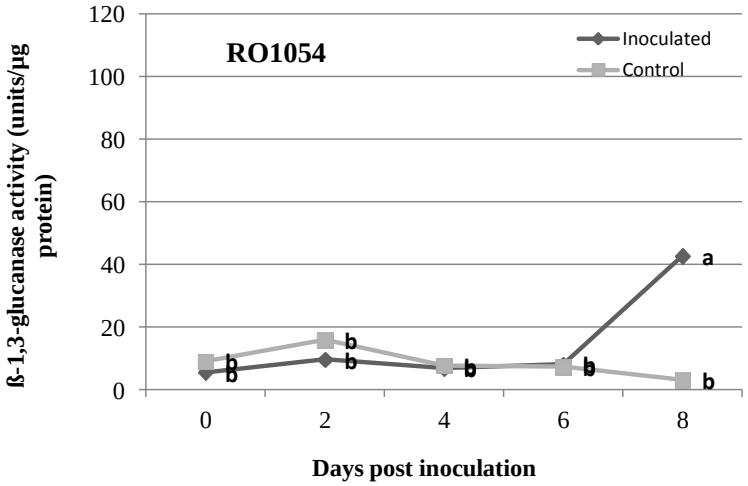
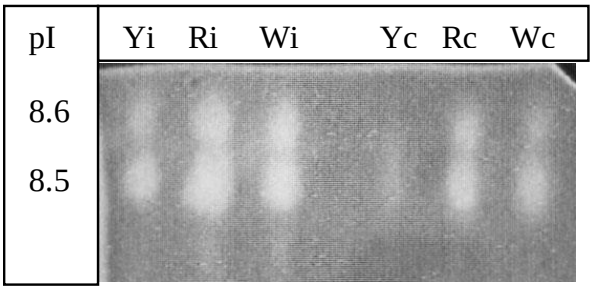
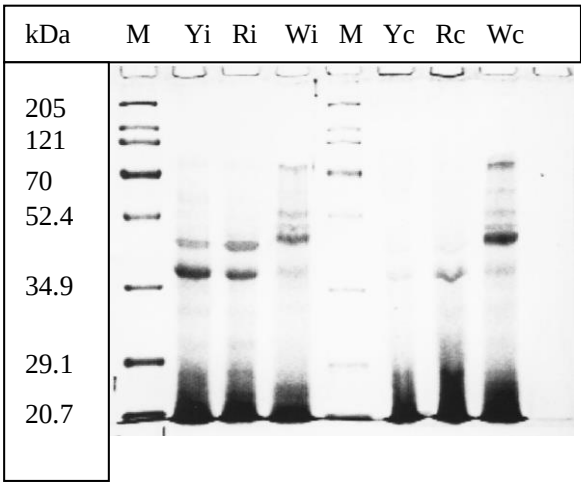



**Figure 3.** β-1,3-glucanase activity in non inoculated roots of cocoyam accessions RO1054 infected with *P. myriotylum*.




**(A)**




**(B)**

**Figure 4.** Chitinase activity in overlay gel following isoelectric focusing of proteins extracted from roots of inoculated cocoyam accessions RO3015 (Yi), RO2063 (Ri), RO1054 (Wi), and those of their respective controls (Yc, Rc, and Wc). The IEF gels contained 4.5 µg protein/well. SDS-PAGE profile of cocoyam proteins extracted from the roots of inoculated RO3015 (Yi), RO2063 (Ri) and RO1054 (Wi) and those of their respective controls (Yc, Rc, Wc).

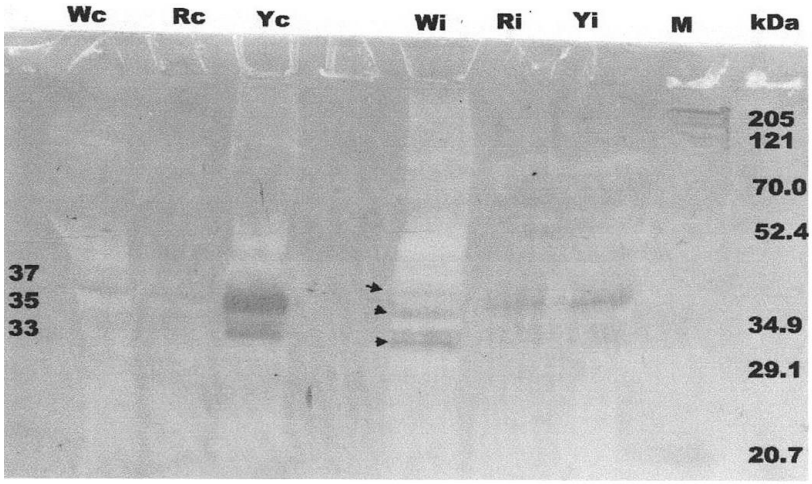



**Figure 5.** Western blot analysis of cocoyam proteins extracted from the roots of inoculated RO3015 (Yi), RO2063 (Ri) and RO1054 (Wi) and those of their respective controls (Yc, Rc, Wc). Proteins were blotted on PVDF membranes following SDS-PAGE and probed with antibodies specific for PR-3 (chitinase).

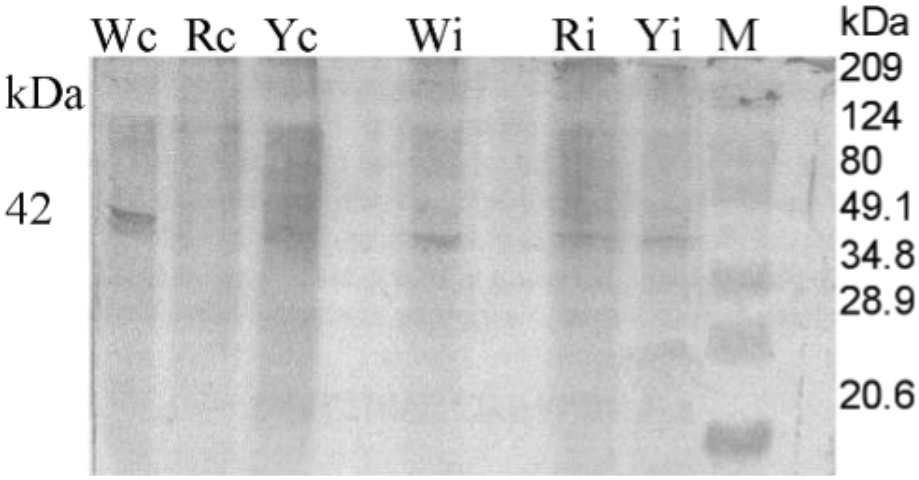



**Figure 6.** Western blot analysis of cocoyam proteins extracted from the roots of inoculated RO3015 (Yi), RO2063 (Ri) and RO1054 (Wi) and those of their respective controls (Yc, Rc, Wc). Proteins were blotted on PVDF membranes following SDS-PAGE and probed with antibodies specific for osmotins.

and characterized the hydrolytic antifungal cell wall degrading enzymes, chitinase and ß-1,3-glucanase including the thaumatin-like PR protein osmotin, induced in cocoyam roots in response to *P. myriotylum* infection.

In the tolerant cocoyam accession RO1054, inoculated plants showed higher chitinase activity in the roots. Both RO1054 and RO3015, which were more tolerant to *P. myriotylum*, also had higher constitutive levels of chitinase in their roots (Figure 2A and C) compared to the susceptible “Local White” and RO2063 (Figure 2B and D). Roots of inoculated RO2063 plants also showed an increase in chitinase activity, however, the increased chitinase activity in this accession did not lead to increased resistance.

ß-1,3-glucanase activity was induced only in RO1054. This activity was relatively low compared to chitinase induction. ß-1,3-glucanase activity was significantly expressed in RO1054 only at 8 dpi. This could be due to a slow accumulation that could possibly increase beyond 8dpi. The low levels of ß-1,3-glucanase activity observed in the tolerant cocoyam (RO1054) suggest that this enzyme may not have a very significant role in cocoyam host defense against *P. myriotylum*. The strong induction of chitinase in the inoculated plants of the same type of cocoyam at 8 dpi compared to the control, could be defense-related in that it may be associated with expres-

sion of induced systemic resistance to any subsequent nonspecific or specific pathogen of cocoyam capable of attacking an already pathogenically challenged cocoyam plant. Tolerance (partial resistance) in RO1054 could be contributed by the synergistic induction of these two hydrolases. In many host-pathogen interactions involving active defense or induced resistance, the pathogenesis-related (PR) endohydrolases with chitinase or β-1,3-glucanase activity, including the thaumatin-like proteins, have been detected in the attacked plant (Anfoka and Buchenauer, 1997; Rahimi et al., 1996; Reiss and Bryngelsson, 1996; Van Loon, 1999). β-1,3-glucanase and chitinase are produced in many active defense responses involving fungi. The substrates for these enzymes are present as structural components of several fungal pathogens. Evidence from *in vitro* studies shows that the action of these enzymes leads to inhibition of fungal growth or hyphal lysis (Mauch et al., 1988; Woloshuk et al., 1991). However, it is unclear why chitinase accumulates in plants attacked by oomycetes since the oomycetes lack chitin in their cell wall. It appears therefore, that the accumulation of chitinase in this host-pathogen interaction is either induced by nonspecific elicitors or part of the induced resistance repertoire, which mainly helps to strengthen the plant's defense system in preparation for subsequent pathogen attack.

In Western analysis, using anti-osmotin polyclonal antibodies from NaCl-adapted tobacco cells, we detected a single protein band (42 kDa) commonly expressed in the roots of the inoculated accessions. Proteins of the PR-5 family have been placed into two major groups based on their cellular localization. Among these proteins, the osmotin-like proteins, which are said to be basic and mainly vacuolar (Jacobs et al., 1999; Kombrink and Somssich, 1995), are known to exhibit antifungal activity (Velazhahan et al., 1999).

This is the first time a study examining induced defense response in cocoyam has been undertaken. Apart from work done on a related aroid (*Colocasia esculenta*) in which cultivars were screened for induction of PR protein in response to infection by *Phytophthora colocasiae* (Ho and Ramsden, 1998), no attempts at understanding the mechanism of cocoyam resistance to the root rot disease have been undertaken at the molecular level.

In this study, we observed a significant increase in chitinase activity in the roots of both tolerant and susceptible cocoyam infected with *P. myriotylum*, however, multiple chitinase bands were expressed only in roots of the tolerant cocoyam. Our results also show that the closely related thaumatin-like (TL) protein, osmotin (PR-5), is induced in cocoyam in response to *P. myriotylum* attack. The increased chitinase and β-1,3-glucanase activity in healthy roots of RO1054 infected with *P. myriotylum* compared to the control, shows that these PRs were systemically induced. Infected roots of inoculated plants were generally avoided in order to exclude pathogen proteins in the analysis. It is apparent, however, that induction of these proteins including osmotins, does not afford complete protection from the invading pathogen. Although osmotins have been shown to be inhibitory to several fungi including the oomycete *Phytophthora infestans* (Woloshuk et al., 1991), it seems members of the PR5 group may be specialized in their functions, for example, possession of antifungal activity against only selective pathogens (Yun et al., 1996). Complete protection from infection would probably require high and rapid accumulation of hydrolytic enzymes and antifungal proteins or accumulation of yet other unidentified antimicrobial compounds in the cocoyam roots. This work only sets the stage for one approach to understanding disease resistance/tolerance in cocoyam to *P. myriotylum*. Revealing the relatively weak defense capability of cocoyams attacked by a virulent isolate of *P. myriotylum* is of interest not only because it increases our understanding of the nature of cocoyam defenses but could offer researchers more rational and viable strategies for the effective control of this pathogen on cocoyam. For example, genetic studies in future research could identify genes in resistant plants expressing high levels of constitutive antifungal proteins that could be engineered into the susceptible consumer preferred cultivars to enhance disease resistance.

## Conflict of Interests

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

## ACKNOWLEDGEMENTS

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