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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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Review

Sulphur oxidising bacteria in mangrove ecosystem: A review

B. C. Behera[1], R. R. Mishra[2], S. K. Dutta[3] and H. N. Thatoi[4]*

[1]Department of Biotechnology, North Odisha University, Baripada -757003, Odisha, India.
[2]Department of Biotechnology, MITS School of Biotechnology, Bhubaneswar-751024, Odisha, India.
[3]Centre for Ecological Sciences, Indian Institute of Science, Bangalore - 560012, India.
[4]Department of Biotechnology, College of Engineering and Technology, Biju Pattnaik University of Technology, Bhubaneswar -751003, Odisha, India.

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Mangrove soils are anoxic, sulphidic and variable since their chemistry is regulated by a variety of factors such as texture, tidal range and elevation, redox state, bioturbation intensity, forest type, temperature and rainfall. Sulphur-oxidizing bacteria such as photoautotrophs, chemolithotrophs and heterotrophs play an important role in the mangrove environment for the oxidation of the toxic sulphide produced by sulphur reducing bacteria and act as a key driving force behind all sulphur transformations in the mangrove ecosystem which is most essential to maintain the sulphur cycle as well as eco health. These overviews summarizes the current state of knowledge of diversity and important biotechnological contributions of these microorganisms in agriculture, bio fertility, reduction of environmental pollution, maintenance of the productivity of ecosystems and also highlight areas in which further research is needed to increase our basic understanding of physiology, genomics and proteomics of these microorganisms which is most essential.

Key words: Mangrove habitat, sulphur oxidising bacteria, sulphur cycle, sulphide oxidase.

INTRODUCTION

Mangroves ecosystems occur in the tropical and subtropical intertidal estuarine region and river deltas of the world. They represent highly dynamic and fragile ecosystems. They are the most reproductive and biologically diversified habitats of various life forms including plants, animals and microorganisms (Holguin et al., 2001). These ecosystems are characterized by periodic tidal flooding which makes the environmental factors such as salinity and nutrient availability highly variable. Mangrove sediments are mainly anaerobic with an overlying thin aerobic sediment layer (Sahoo and Dhal, 2009). Degradation of organic matter in the aerobic zone occurs by various microorganisms and among various microorganisms, bacteria play major roles in the chemical and biological redox reactions in this ecosystem that create the biogeochemical cycle. Among the various biogeochemical cycles that takes place in this rich detritus based coastal sediment; the sulphur cycle

*Corresponding author. E-mail: hn_thatoi@rediffmail.com. Tel: +91-9437306696.

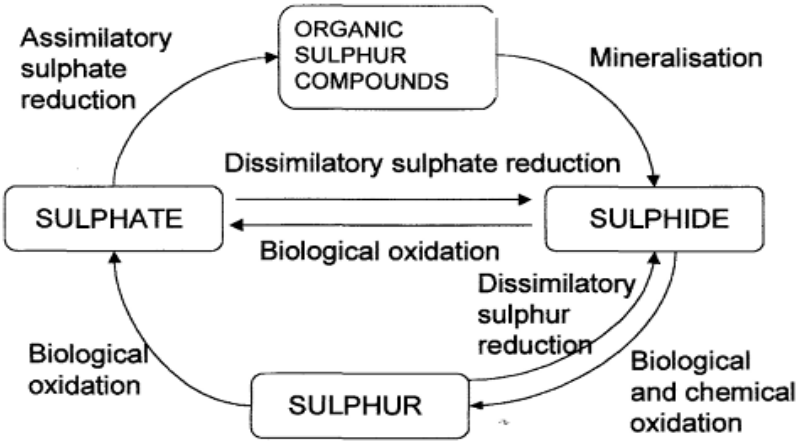

Figure 1. The biological sulphur cycle (Janssen et al., 1999).

(Figure 1) is one of them. Sulphur is biochemically very significant. It is utilized for its structural and functional role in the amino acids cysteine and methionine, and in vitamins such as thiamine, biotin and lipoic acid, as well as in coenzyme A (Madigan et al., 2000).

Sulphur oxidation improves soil fertility. It results in the formation of sulphate, which can be used by the plants, while the acidity produced in sulphur oxidation helps to solubilize plant nutrients and lowers the pH of alkaline soils (Wainwright, 1984). In addition, the sulphur cycle is closely linked to other element cycles, such as the carbon and nitrogen cycles. In the anaerobic layer decomposition occurs mainly through sulphate-reduction (Nedwell et al., 1994). Sulphur-oxidizing bacteria play an important role in the detoxification of reduced sulphide in sediments. The decomposition of organic substance involves various trophic groups of microorganisms acting in a multi-step process. The first step is an enzymatic hydrolysis of polymeric material to soluble monomeric and oligomeric compounds. Under oxic conditions the soluble compounds are directly mineralised to carbon dioxide and water. Under anoxic conditions various physiological groups are involved in the degradation after the initial depolymerisation. Fermentative bacteria convert the products of hydrolysis to a variety of products, mainly short chain fatty acids, carbon dioxide and hydrogen ion (Das et al., 2012). Further conversion through the action of secondary fermenters, sulphate-reducers, acetogens and methanogens produces the end products CO_2, CH_4 and H_2S which may escape into the atmosphere (Das et al., 2012). All three are important greenhouse gases. The extent of the fluxes depends on the penetration of oxygen and the activity of aerobic bacteria in the surface layer, which can oxidize sulphide and methane. Hydrogen sulphide can be oxidised to elemental sulphur, thiosulphate or sulphate (Lyimo et al., 2002). Hydrogen sulphide also precipitates easily with metal ions as the corresponding metal sulphide, for instance FeS, which gives many anoxic types of sediment their black colouration (Lyimo et al., 2002).

The literature shows that mangrove soils are sulphidic and variable, since their chemistry is regulated by a variety of factors such as texture, tidal range and elevation, redox state, bioturbation intensity, forest type, temperature and rainfall (Alongi, 1992). Although several papers on the diversity of sulphur reducing bacteria on the mangrove micro biota have been published, the knowledge of sulphur oxidising bacterial communities and their genomics, metagenomics and proteomics studies with reference to sulphur oxidation in mangrove sediments is sparse. The present review is an attempt to consolidate the latest studies and critical research on diversity of sulphur oxidising bacteria in mangrove and to showcase their important contribution towards the biogeochemical cycle of the ecosystem.

SULPHUR CYCLE IN MANGROVE HABITAT

Sulphur-oxidizing bacteria play an important role in the detoxification of sulphide in water and sediments. Symbiotic sulphur-oxidizers, for example, those within members of the bivalve family Lucinacea, can be commonly found in muddy mangrove areas (Liang et al., 2006). Sulphur reducing bacteria are anaerobic micro-organisms that are wide spread in anoxic habitats like mangrove, where they use sulphate as a terminal electron acceptor for the degradation of organic compounds, resulting in the production of sulphide. Subsequently, the sulphide can be oxidized under oxic conditions by chemolithootrophic sulphur bacteria or under anoxic conditions by phototrophic sulphur bacteria. Sulphur oxidising chemolithotrophs growth is primarily aerobic, that is, using molecular oxygen as terminal

electron acceptor. However, some species (*Beggiatoa* sp., *Thioploca* sp., *Thiobacillus denitrificans*, *Thiomicrospira denitrificans*) oxidize H_2S and aerobically coupling it to nitrate reduction (Brock et al., 2006). In salt marshes, the ecological equivalent of mangroves in temperate areas, sulphate reduction is known to be the major mineralization process. The large inputs of organic matter support high rates of heterotrophic metabolism. Since oxygen is usually depleted below a few millimetre depths, even where the sediment surface is exposed to air, anaerobic metabolism predominates with decomposition mediated primarily by fermentative and sulphate reducing bacteria (King, 1988). Sulphide formed as the product of bacterial sulphate reduction usually undergoes rapid digenetic transformations in coastal sediments. Hence, microbial sulphur transformation is a key process for the biogeochemical sulphur cycle in marine sediments and closely linked to the cycling of other elements like oxygen, nitrogen, and carbon (Bruser et al., 2000).

The major processes of transformation involved in the cycling of sulphur in the environment are:

1. Mineralization of organic sulphur to the inorganic form, hydrogen sulphide, H_2S.
2. Immobilization
3. Oxidation and
4. Reduction

Mineralization

The breakdown/decomposition of large organic sulphur compounds to smaller units and their conversion into inorganic compounds (sulphates) by the microorganisms.

Immobilization

Immobilization involves microbial conversion of inorganic sulphur compounds to organic sulphur compounds. In the process of immobilization, microorganisms absorb inorganic sulphate and convert it into organic form for the synthesis of microbial tissue. If an abundant supply of carbon is available for energy then the entire inorganic sulphate in soil will be converted to organic form, but if little carbon is available then inorganic sulphate will be released from the organic matter. Plant absorbs inorganic sulphate and converts it into organic sulphur compound (Subba Rao, 1999).

Oxidation

Sulphate on the reductive side functions as an electron acceptor in metabolic pathways is used by a wide range of microorganisms and is converted to sulphide. Reduced sulphur compounds such as sulphide serve as electron donors for phototrophic or chemolithothrophic bacteria which convert these compounds to elemental sulphur or sulphate (Robertson and Kuenen, 2006) (Eq^n...1 and 2). When plant and animal proteins are degraded, sulphur is released and accumulates in the soil which is then oxidized to sulphates in the presence of oxygen and under anaerobic condition (water logged soils) organic sulphur is decomposed to produce hydrogen sulphide (H_2S) (Eq^n...3). H_2S can also accumulate during the reduction of sulphates under anaerobic conditions which can be further oxidized to sulphates under aerobic conditions.

$$2S + 3O_2 + 2H_2O \longrightarrow 2H_2SO_4 \longrightarrow 2H(+) + SO_4 \text{ (Aerobic)} \quad (1)$$

$$CO_2 + 2H_2S \xrightarrow{\text{Light}} (CH_2O) + H_2O + 2S \quad (2)$$

$$OR\ H_2 + S + 2CO_2 + 2H_2O \xrightarrow{\text{Light}} H_2SO_4 + 2(CH_2O) \text{ (anaerobic).} \quad (3)$$

The biological oxidation of elemental sulphur and inorganic sulphur compounds (such as H_2S, sulphite and thiosulphate) to sulphate (SO_4) is brought about by direct and indirect methods. In the direct approach photoautotrophic or chemolithotrophic sulphide oxidizing bacteria use sulphide as an electron donor and convert it to sulphur or sulphate. Photoautotrophs use CO_2 as the terminal electron acceptor, while with chemolithotrophs, oxygen (aerobic species) or nitrate and nitrite (anaerobic species) serve as terminal electron acceptors. In the indirect method oxidation of reduced sulphur compound is carried out chemically by ferric iron as the oxidizing agent, and iron oxidizing bacteria are used to regenerate the ferric iron for further use (Pagella and De Faveri, 2000).

Photoautotrophic oxidation of sulphide

The bulk of hydrogen sulphide formed by dissimilatory sulphur reduction is most probably oxidised to sulphate by the respiratory activity of various aerobic sulphur oxidising bacteria and by the direct reaction with oxygen resulting in several intermediary oxidation products such as, sulphur and thiosulphate (Kuenen, 1975). Phototrophic oxidation of sulphide is an anaerobic process which is carried out by green sulphur bacteria such as *Chlorobium,* and purple sulphur bacteria such as *Allochromatium* (Madigan and Martinko, 2006). These bacteria utilize H_2S as an electron donor for CO_2 reduction in a photosynthetic reaction referred to as the vanNiel reaction as described in (Eq^n...4) (Janssen et al.,

1999). Under special condition, however when light has access to the anaerobic, sulphide containing water or the sediment surface, anaerobic phototrophic bacteria may develop which oxidise sulphide and sulphur to sulphate with the concomitant reduction of carbon dioxide to cell substances. The phototrophic green sulphur bacteria, Chlorobiaceae, possess with their chlorosomes, the most efficient light harvesting system, which allows them to grow at lower light intensities or at the lower layer of the water level, adjacent to the sulphide production zone. They oxidise the available sulphide to elemental sulphur outside the cell which is further oxidised to sulphate or reduced to sulphide by sulphur reducing bacteria.

$$2H_2S + CO_2 + \text{Light} \longrightarrow 2S + CH_2O\,(\text{carbohydrate}) + H_2O \quad (4)$$

The purple sulphur bacteria encompass many genera such as *Chromatium*, *Thioalkalicoccus*, *Thiorhodococcus*, *Thiocapsa*, *Thiocystis*, *Thiococcus*, *Thiospirillum*, *Thiodictyon*, and *Thiopedia.* Some of the genera *Ectothiorhodospira*, *Thiorhodospira* and *Halorhodospira* showed special interest because unlike other purple sulphur bacteria, the sulphur produced by these bacteria resides outside the cell (Madigan and Martinko, 2006). Although light seems to be the main source of energy for photoautotrophic sulphide oxidizers, lithoautotrophic growth in the absence of light has been documented for certain purple sulphur bacteria such as *Allochromatium vinosum* and *Thiocapsa roseopersicina* (Friedrich et al., 2001). Green sulphur bacteria, encompassing key genera such as *Chlorobium*, *Prosthecochloris*, *Pelodictyon*, *Ancalochloris, Rhodopseudomonas* and *Chloroherpeton*, use H_2S as an electron donor, oxidizing it first to elemental sulphur and then to sulphate (Tang et al., 2009).

Chemolithotrophic sulphide oxidation

The chemolithotrophic sulphide oxidizers also referred to as colourless sulphur bacteria, do not contain bacteriochlorophyll. In terms of energy and carbon sources, the colourless sulphide oxidizers are classified into four groups. (i) Obligate chemolithotrophs need an inorganic source for energy, and use CO_2 as their carbon source. Despite the classification as "obligate" autotrophs, many species have been shown to benefit from small amount of supplemented carbon compounds (Matin, 1978). Many species of *Thiobacillus*, at least one species of *Sulfolobus*, and all of the known species of *Thiomicrospira* belongs to this category (Tang et al., 2009; Kuenen, 1975). (ii) Facultative chemolithotrophic sulphide oxidizers can grow either chemolitho-autotrophically with carbon dioxide and an inorganic energy source, or heterotrophically with complex organic compounds as carbon and energy source, or mixotrophically using both pathways simultaneously (Tang et al., 2009). Some species of *Thiobacilli*, *Thiosphaera pantotropha*, *Paracoccus denitrificans* (Friedrich and Mitrenga, 1981) and certain *Beggiatoa* (Nelson and Jannasch, 1983) are typical examples of facultative chemolithotrophic sulphide oxidizers. (iii) Chemolithoheterotrophs are characterized by the ability to generate energy from oxidation of reduced sulphur compounds, while being unable to fix CO_2 (Tang et al., 2009). A few species of *Thiobacillus* and some *Beggiatoa* strains fall into this category. (iv) Chemoorgano-heterotrophs such as *Thiobacterium* and *Thiothrix* and some species of *Beggiatoa* can oxidize reduced sulphur compounds without deriving energy from them. These organisms use this reaction as means for detoxifying the metabolically produced hydrogen peroxide (Larkin and Strohl, 1983). They have diverse morphological, physiological and ecological properties and are able to grow chemolithotrophically on reduced inorganic sulphur compounds such as sulphide, sulphur and thiosulphate and in some cases organic sulphur compounds like methanethiol, dimethylsulphide and dimethyldisulphide (Madigan and Martinko, 2006). Many sulphur chemolithotrophs are aerobic as the terminal electron acceptor is primarily oxygen. However, some species can grow anaerobically using nitrate or nitrite as the terminal electron acceptor.

The colourless sulphur bacteria encompass many genera such as *Thiobacillus*, *Acidithiobacillus*, *Achromatium*, *Beggiatoa*, *Thiothrix*, *Thioplaca*, *Thiomicrospira*, *Thiosphaera*, and *Thermothrix* etc. *Achromatium*, a spherical sulphur oxidizer, is common in fresh water sediments containing sulphide. Similar to *Chromatium*, *Achromatium* store elemental sulphur internally as granules which eventually disappear as sulphur is further oxidized to sulphate (Madigan and Martinko, 2006). The genus *Thiobacillus*, one of the most studied groups, consists of several Gram negative and rod shaped species which utilize oxidation of sulphide, sulphur and thiosulphate for generation of energy and growth (Robertson and Kuenen, 2006). The members of genus *Thiobacillus* (obligate chemolithotrophic, non photosynthetic) for example, *T. ferrooxidans* and *T. thiooxidans* are the main organisms involved in the oxidation of elemental sulphur to sulphates. These are aerobic, non-filamentous, chemosynthetic autotrophs. The *thiobacilli* can also oxidize thiosulphate (Eq^n...5).

$$Na_2S_2O_2 + O_2 + HOH \longrightarrow Na_2SO_4 + H_2SO_4 \quad (5)$$

And tetrathionate--(Eq^n...6)

$$2Na_2S_4O_6 + 7O_2 + 6HOH \longrightarrow 2Na_2SO_4 + 6H_2SO_4 \quad (6)$$

Oxidation of reduced sulphur compounds generates significant acidity and thus several species of *Thiobacillus*

are acidophilic. One such species, *Acidithiobacillus ferrooxidans* can also grow chemolithotrophically by the oxidation of ferrous iron (Eq^{n}...7). *Acidithiobacillus ferrooxidans* - an acidophile, very tolerant of low pH (pH between 1 and, at least, pH 5.5) (Quatrini et al., 2003). In addition to oxidizing hydrogen sulphide, this organism can extract iron (Eq^{n}...8) from solid pyrite (FeS_2) in a two-step process in which sulphur atoms are oxidized. First, the organism catalyzes the oxidation of ferrous iron, generating ferric iron

$$Fe^{2+} + 1/2\ O_2 + 2\ H^+ \longrightarrow Fe^{3+} + H_2O \quad (7)$$

Secondly, the ferric iron produced spontaneously reacts with pyrite

$$FeS_2 + 14\ Fe^{3+} + 8\ H_2O \longrightarrow 15\ Fe^{2+} + 2\ SO_4^{2-} + 16\ H^+ \quad (8)$$

The reaction is self-supporting, since the ferrous iron produced in the second reaction can be fed back into the first reaction. Heterotrophic bacteria (*Bacillus*, *Pseudomonas*, and *Arthrobacter*) and fungi (*Aspergillus, Penicillium*) and some actinomycetes were also reported to oxidize sulphur compounds. The important reactions involved in chemolithotrophic oxidation of sulphide, sulphur and thiosulphate under aerobic conditions can be summarized as (Madigan and Martinko, 2006).

$$H_2S + 1/2O_2 \longrightarrow S + H_2O \quad (9)$$

$$S + 3/2O_2 + H_2O \longrightarrow SO_4^{2-} + 2H^+ \quad (10)$$

$$H_2S + 2O_2 \longrightarrow SO_4^{2-} + 2H^+ \quad (11)$$

$$S_2O_3^{2-} + H_2O + 2O_2 \longrightarrow 2SO_4^{2-} + 2H^+ \quad (12)$$

Various colourless sulphur bacteria grow differently under anaerobic conditions, one of the best known pathways is the use of nitrate or nitrite as terminal electron acceptors. Oxidation of sulphide under denitrifying conditions could lead to formation of sulphur, sulphate and nitrite or nitrogen based on the following reactions (Cardoso et al., 2006).

$$S^{2-} + 1.6NO^{3-} + 1.6H^+ \longrightarrow SO_4^{2-} + 0.8N_2 + 0.8H_2O \quad (13)$$

$$S^{2-} + 0.4NO^{3-} + 2.4H^+ \longrightarrow S + 0.2N_2 + 1.2H_2O \quad (14)$$

$$S^{2-} + 4NO^{3-} \longrightarrow SO_4^{2-} + 4NO^{2-} \quad (15)$$

$$S^{2-} + NO^{3-} + 2H^+ \longrightarrow S^- + NO^{2-} + H_2O \quad (16)$$

Oxidation of sulphur and thiosulphate under denitrification can be represented by the following reactions.

$$S + 1.2NO3 - + 0.4H_2O \longrightarrow SO4^{2-} + 0.6N_2 + 0.8H^+ \quad (17)$$

$$S_2O_3^{2-} + 1.6NO_3^- + 0.2H_2O \longrightarrow 2SO4^{2-} + 0.8N_2 + 0.4H^+ \quad (18)$$

Reduction

Two physiological types of sulphate reduction are recognized **(**Peck, 1961**)**. The first is assimilatory or biosynthetic sulphate reduction in which organisms reduces only enough sulphates to meet their nutritional requirements for sulphur. The assimilatory pathway generates reduced sulphur compounds for biosynthesis of amino acids and proteins. This pathway is considered to be in the pathway for the biosynthesis of cysteine and is usually under both coarse and fine metabolic regulation (Roy and Trudinger, 1970)**.**

The second sequence involved in the reduction of sulphate is the dissimilatory or respiratory pathway of sulphate reduction in which sulphate in the absence of oxygen serves as a terminal electron acceptor for anaerobic respiration. Sulphate can be reduced to hydrogen sulphide (H_2S) by sulphate reducing bacteria (for example, *Desulfovibrio* and *Desulfatomaculum*) and may diminish the availability of sulphur for plant nutrition. This is “dissimilatory sulphate reduction” which is not at all desirable from soil fertility and agricultural productivity view point. Dissimilatory sulphate-reduction is favoured by the alkaline and anaerobic conditions of soil and sulphates are reduced to hydrogen sulphide. For example, calcium sulphate is attacked under anaerobic condition by the members of the genus *Desulfovibrio* (Eq^{n}...19).

$$CaSO_4 + 4H_2 \longrightarrow Ca\ (OH)_2 + H_2S + 2\ H_2O \quad (19)$$

Hydrogen sulphide produced by the reduction of sulphate and sulphur containing amino acids decomposition is further oxidized by some species of green and purple phototrophic bacteria (*Chlorobium, chromatium*) to release elemental sulphur (Eq^{n}...20).

$$CO_2 + 2H_2 + H_2S \xrightarrow{\text{Light}} (CH_2O) + H_2O + 2\ S \quad (20)$$

In dissimilative sulphur reduction, sulphate acts as a terminal electron acceptor during the energy-generating oxidation of various materials.

DIVERSITY OF SOB IN DIFFERENT MANGROVE ECOSYSTEM

The knowledge of sulphur oxidising bacterial (SOB)

communities in mangrove sediments is sparse. There are some reports on diversity of SOB from mangrove environment. Sulphur rich mangrove ecosystem, which have mainly anaerobic soil environment, provide favourable condition for the proliferation of photosynthetic anoxygenic bacteria such as purple sulphur bacteria (family *Chromatiaceae*, strain belongs to *Chromatium* sp.) and purple non sulphur bacteria (family *Rhodospirilaceae*, strain belongs to *Rhodopseudomonas* sp.) (Sahoo and Dhal, 2009). Purple sulphur bacteria range in colour from pink to purple and contain bacterio chlorophyll *a* as their major pigment. Purple sulphur bacteria are widely distributed in sulphide rich reducing environment such as mangrove habitat, mud flat and polluted water. These phototrophic anaerobes require sulphide which they oxidise to sulphate for their growth. Physiologically family Chromatiaceae which contain sulphur globules inside their cells are able to oxidise sulphur further to sulphate (Pfenning, 1977). Representatives of the family Chromatiaceae and Rhodospirilaceae were also previously reported from the Indian mangrove habitat (Vethanayagam, 1991; Vethanayagam and Krishnamurthy, 1995) Strains belonging to *Chromatium* sp. *(*Family Chromatiaceae-purple sulphur bacteria) and *Chloroflexux* sp. (family Chloroflexaceae-micro filamentous green photosynthetic bacteria) were also reported to occur in the mangrove habitat (Krishnamurthy et al., 1986). Thatoi et al. (2012) reported the occurance of *Pseudomonas* sp., which oxidised sulphur in the mangrove soil of Bhitarakanika, Odisha, India. The predominant sulphur oxidising bacteria in the mangrove ecosystem of Cochin were identified as members of the genera *Chloronema, Chromatium, Beggiatoa, Thiopedia* and *Leucothiobacteri* (Dhevendaran, 1991). Large population of *chromatium* grew in enrichment culture made of Florida's mangrove sediment (Sahoo and Dhal, 2009). In sediment from the Egyptian coast of the Red Sea on which mangroves grew, 225 isolates of purple non sulphur bacteria belonging to ten species, representing four different genera, were identified. The most common genera were *Rhodobacter* and *Rhodopseudomonas* (Shoreit et al., 1994).

The bacterial diversity present in sediments of a well-preserved mangrove habitat in Ilha do Cardoso, located in the extreme south of Sa˜o Paulo State coastline, Brazil, was assessed using culture independent molecular approaches (denaturing gradient gel electrophoresis (DGGE). The data revealed that the gamma-proteobacteria present were 19.28% of the total bacterial community (Dias et al., 2010). The representatives of these Gamaproteobacterial genera were *Acidithiobacillus, Alkalilimnicola, Frateuria, Fulvimonas, Shewanella, Thiorhodospira,* and *Thiobacillus*. *Marichromatium* belonging to the class gammaproteobacteria also reported from a marine Indian aquaculture pond by Kumar et al. (2007). Some of the sulphur oxidising bacteria such as gammaproteobacteria for example, *Chromatiales* were also reported from oil contaminated soil of Brazilian pristine mangrove sediment (Holguin et al., 2001). The Proteobacteria was the most abundant phylum and metabolically highly diverse, widely distributed in marine environments and is an important player in nutrient cycling (Kersters et al., 2006). The potential effect of mangrove roots on sediment proteobacterial populations may influence several environmentally relevant processes in mangrove ecosystems. Gomes et al. (2010) observed that Chromatiales was the second most abundant proteobacterial order and was detected in all samples from an urban mangrove habitat located in Guanabara Bay, Rio de Janeiro, Brazil. This order is represented by anaerobic or microaerophilic microorganisms specialized in sulphur-an oxygenic photosynthesis and are able to oxidize hydrogen sulphide (H_2S) to elemental sulphur (Imhoff, 2006). Campylobacterales were also abundant and mainly detected in the mangrove samples with a marked increased abundance in rhizosphere samples. Members of this order are sulphide-oxidizing denitrifying bacteria (Campbell et al., 2006). *Sulphurovum* belonging to the order Campylobacterales was reported in mangrove rhizosphere samples. This genus is known to be an important player in the process of sulphide-oxidation and denitrification in marine environments (Sievert et al., 2008). The genus *Listonella* includes diazotrophic members with some representative previously detected from mangrove rhizosphere (Gomes et al., 2010). Hart (1958; 1959; 1962) demonstrated that mangrove peat contains large amounts of sulphides and polysulphides. He showed that free sulphur was oxidized to sulphuric acid by *Thiobacillus thio-oxidans* from tidal mangrove soil of Sierra Leone. *Thiobacillus thio-oxidans* was also reported from the mangrove swamp of Keneba (Thornton and Giglioli, 1965). The gammaproteobacteria represented the most abundant proteobacterial subdivision (59% and 77%) among the proteobacterial division reported from Sundarban mangrove habitat, India. The most abundant genera reported are methylophaga, indicating a strong involvement of these bacterial species in the maintenance of the biogeochemical cycle in Sundarban sediment (Ghosh et al., 2010). Kaambo (2006) reported that γ-*Proteobacteria* are the second dominant bacterial group which was observed at the upstream site of the sediments of the Great Berg River estuary of South Africa. They oxidize sulphide to sulphur and are often found in anaerobic sulphur rich regions (Holmer et al., 2001). The gammaproteobacteria such as *Thioalkalivibrio nitratireducens, Thioalkalivibrio denitrificans, Rhabdochromatium marinum*, and *Thiococcus* sp. SZB80 (related (94.9% similarity) to *R. marinum*, a purple sulphur bacterium (Dilling et al., 1995) reported from Futian mangrove swamp) were phylogenetically

associated with cultivated organisms involved in S- or N-cycles (Liang et al., 2006).

Symbiotic association of sulphur-oxidizing bacteria with other organisms has also been reported to occur in mangrove environments. Four tropical lucinids, *Codakia orbiculata, C. pectinella, Linga pensylvanica,* which inhabit sea-grass beds, and *Lucina pectinata*, which inhabits mangrove swamps in Guadeloupe, harbour sulphur-oxidizing endosymbiotic bacteria within bacteriocytes of their gill filaments (Gros et al., 1998). Some of the free-living and symbiotic sulphur oxidising bacteria for example, those within members of the bivalve family Lucinacea were reported from Futian mangrove swamp of China (Liang et al., 2006).

ENZYME RESPONSIBLE FOR SULPHUR OXIDATION

Many published reports address microbial sulphide oxidation. Sulphide oxidase is the key enzyme responsible for sulphide ions oxidation (Mohapatra et al., 2006). Sulfite oxidase contains two identical subunits. The N-terminal domain has a heme cofactor with three adjacent antiparallel beta sheets and five alpha helices. The C-terminal domain hosts a molybdopterin cofactor that is surrounded by thirteen beta sheets and three alpha helices. The molybdopterin cofactor has a Mo (VI) center, which is bonded to sulphur from cysteine, an ene-dithiolate from pyranopterin and two terminal oxygens.

Sulphide oxidase which catalyzes the oxidation of sulphide has been characterized from *Arthrobacter* sp. and *Bacillus* sp. BN53-1 (Mohapatra et al., 2006; Nakada and Ohta, 1999). According to Mohapatra et al. (2006) report, the purified sulphide oxidase was showed to be monomer with a molecular weight of 43 kDa. This molecular weight was found to be higher compared to the purified enzyme from the *Bacillus* sp. BN53-1 which is 37 kDa (Nakada and Ohta, 1999). The sulphide oxidase isolated from *Arthrobacter* sp. was cell-bound and had broad pH activities which are potentially useful in application of the wastewater treatment process (Mohapatra et al., 2006). To our knowledge, in addition to sulphide oxidase there are some other enzyme that are also responsible for sulphur oxidation. Such as, the involvement of *Serratia* regarding sulphate formation from the hydrolysis of organic sulphate by enzymes termed sulphatase (Murooka et al., 1980).

SOX GENE IN SULPHUR OXIDISING BACTERIA

The genes encoding sulphur-oxidizing (Sox) ability is known as *Sox* gene cluster was first described from the alphaproteobacterium *Paracoccus pantotrophus* which is a facultative *chemolithoatotrophs* and grows with thiosulphate (Friedrich et al., 2001). The *sox* gene cluster encoding multienzyme Sox complex of *Pcs. pantotrophus* comprises of at least two transcriptional units of 15 genes (*soxRSVWXYZABCDEFGH*).

SoxR encodes a repressor protein, of ArsR family SoxR which binds to the soxS–V and soxW–X intergenic regions of the gene (Figure 2). SoxS is a periplasmic thioredoxin and essential for the full expression of the *sox* gene cluster (Rother et al., 2005). soxV encodes a membrane protein and soxW encodes a periplasmic thioredoxin. Both are essential for chemotrophic growth with thiosulphate and are probably involved in transfer of reluctant (Bradischewsky and Friedrich, 2001). The subsequent genes *soxXYZABCD* encode four periplasmic proteins, SoxXA, SoxYZ, SoxB and Sox (CD) 2, which reconstitute the core Sox enzyme system. These seven gene *soxXYZABCD,* code for proteins essential for sulphur oxidation *in vitro* (Ghosh et al., 2009) (Figure 2). Sox*EFGH* gene are located downstream of sox*D*. These genes are co expressed with sox structural gene. SoxXA is composed of two *c*-type cytochromes, the diheme SoxX and the monoheme SoxA. SoxYZ is free of cofactors and able to covalently bind sulphur compounds of various oxidation states (Quentmeier and Friedrich, 2001). The SoxB protein, which contains a dinuclear manganese cluster was proposed to function as a sulphate thiohydrolase and has been shown to interact with the SoxYZ complex (Quentmeier et al., 2003). SoxB is believed to act as a sulphate thiol esterase and to be responsible for hydrolytic cleavage of a sulphate group from the bound sulphur substrate. Sox (CD) 2 then oxidize the remaining sulphane sulphur, acting as a sulphur dehydrogenase. Sox (CD) 2 are composed of the molybdoprotein SoxC and the diheme *c*-type cytochrome SoxD (Quentmeier et al., 2000). SoxF gene encodes the monomeric flavoprotein SoxF that has sulphide dehydrogenase activity (Bardischewsky et al., 2005). A novel activity has been discovered for SoxF to activate the thiosulphate- or sulphide-oxidizing Sox enzyme system when reconstituted with a SoxYZ protein separately inactivated by reduction.

Genes homologous to those encoding Sox proteins of *P. pantotrophus* (Figure 2) have been identified from partially and completely sequenced genomes of other sulphur oxidising bacteria. For example the sox cluster gene of *Rhodopseudomonas palustris* is similar to that of *P. Pantotrophus* and is composed of 16 gene. The Chlorobiaceae are anoxygenic phototrophic green sulphur bacteria that oxidize hydrogen sulphide to sulphuric acid and transiently deposit sulphur globules outside the cell. The genome of *Chlorobium tepidum*, a moderate thermophile, contains a cluster of 13 genes of which soxFXYZAB are homologous to the respective genes of *P. pantotrophus*. *A. Aeolicus* is an obligatory aerobic chemolithotrophic bacterium. This organism requires molecular hydrogen for lithoautotrophic growth and does not grow with thiosulphate alone. The *sox* gene

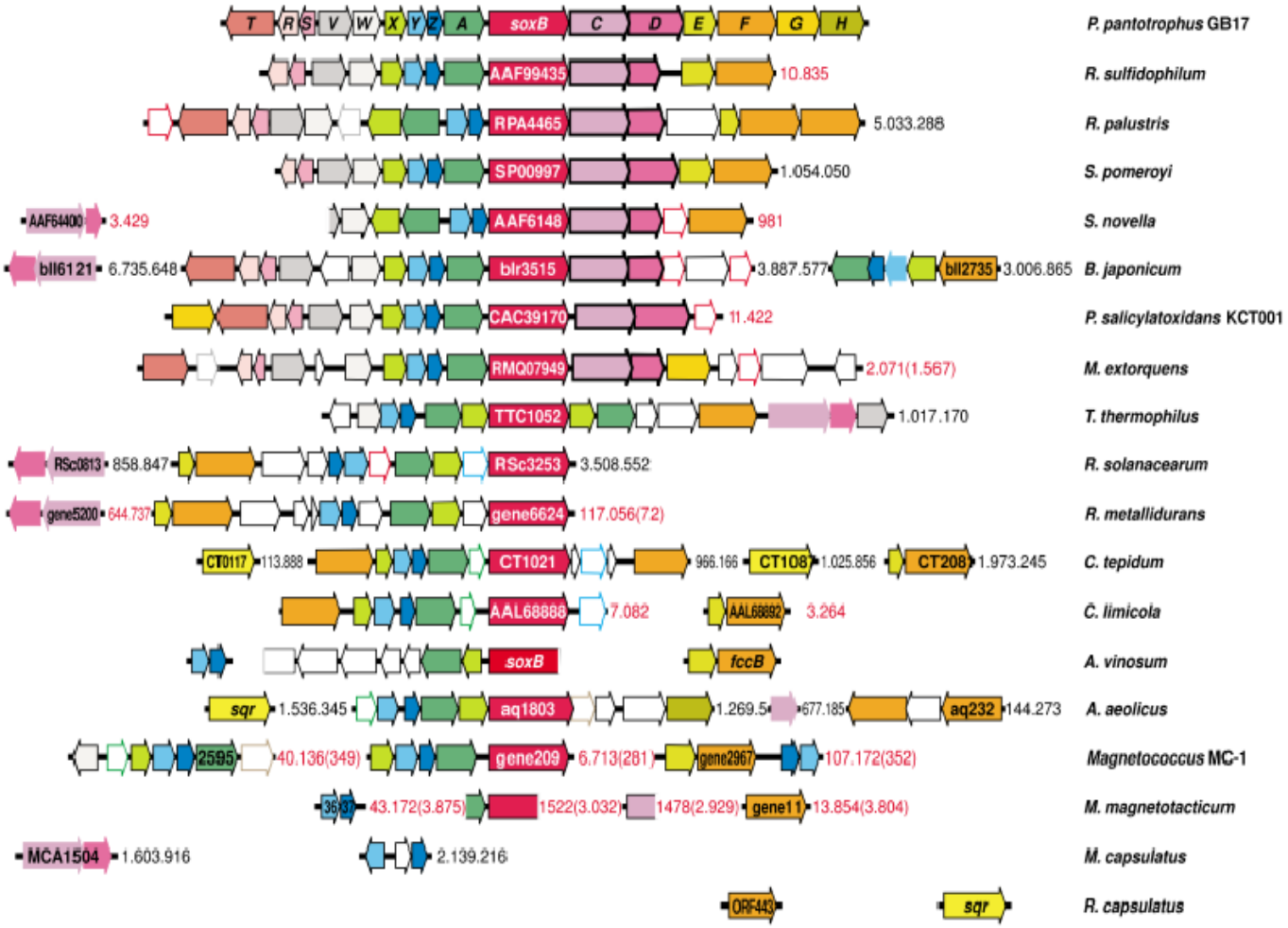

Figure 2. Schematic overview of the sox-locus of *P. Pantotrophus* and related gene of chemolithotrophic and phtotrophic bacteria. (Friedrich et al., 2005).

cluster of *A. Aeolicus* comprises 10 genes. Besides that the complete sox gene clusters encoding essential components of the Sox enzyme system in *P. pantotrophus* are present in partially sequenced genomes of chemotrophic bacteria such as *S. novella*, *Methylobacterium extorquens, Pseudaminobacter salicylatoxidans* and *Bradyrhizobium japonicum*. Also, complete *sox* gene clusters are present in the phototrophs *R. sulfidophilum, Rhodopseudomonas palustris* and the chemolithoheterotroph *Silicibacter pomeroyi*

SOME IMPORTANT BIOLOGICAL APPLICATIONS OF SULPHUR OXIDIZING BACTERIA

Bio leaching

At acid pH, *Thiobacillus ferrooxidans* uses ferrous iron as its energy source and produces ferric iron. This reaction is of great importance in the formation of acid leachate from mining operation and in the microbial leaching of metals from ores. Both processes involve microbiological and chemical reactions. Under aerobic and acidic conditions, *T. ferrooxidans* oxidizes ferrous to ferric iron. Ferric iron chemically oxidizes pyrite to form more ferrous iron and sulphuric acid. The bioleaching, biooxidation of metal sulphides to soluble metal sulphates and sulphuric acid is affected by specialized bacteria. Three species of mesoacidophilic, chemolithotropic bacteria are mainly involved. *Thiobacillus ferroxidants*, *Thiobacillus thiooxidants* and *Leptospirillum ferroaxidans*, *Thiobacillus ferroxidans* oxidizes reduced sulphur compounds to sulphate and iron (II) to iron (III) ions. *Thiobacillus thiooxidants* is able to oxidize only reduced sulphur compounds whereas *L. ferroaxidants* can oxidize only iron (II) ions (Schippers and Sand, 1999).

Biofertiliser

Thiobacilli can also be used in the manufacture of a form of organic fertilizer long favored in Australia. In 'biosuper', a mixture of rock phosphate and sulphur is inoculated with *Thiobacillus thiooxidans.* The biochemical oxidation of the sulphur produces H_2SO_4 which decreases soil pH and solubilizes $CaCO_3$ in alkaline calcareous soils to make soil condition more favourable for plant growth, including the availability of plant nutrients (Linderman et al., 1991), especially phosphorous (Deluca et al., 1989). Applying biofertilizers that is, *mycorrhizae* and *Thiobacillus* that also increased soybean yield has been reported. Symbion-S is a liquid form of “Bio-fertilizer” based on selective strain of sulphur solubilizing bacteria, *Thiobacillus thiooxidans.* These beneficial bacteria are suspended liquid carrier, suspended liquid carrying 10^9 bacterial cells/ml of the product. The bacteria used for the production of this product, namely *Thiobacillus thiooxidants* strain are known for its sulphur solubilizing characters. This bacterial cell converts the non available sulphur and sulphur related compounds to easily assimilable form of sulphur salts through a process of oxidation. During this process, it brings down the high pH of the soil (alkasol soil). Hence, Symbion-S can be utilized in reclaiming the alkaline and saline soil for normal cultivation.

Plant growth promotion

The use of *Rhizobium* inoculant for better crop production is a common practice in agriculture which allows the legume plants to form root nodules within which atmospheric nitrogen is fixed and supplied to the plant. It has also been reported that the synergistic response that occurs when a sulphuroxidizing plant growth promoting rhizobacteria (PGPR) *Delftia acidovorans* RAY209 is added to some *Rhizobium* inoculants, the plants showed enhanced seed emergence, increased biomass, and increased nodule numbers (Yesmin et al., 2004). A number of sulphur oxidizing plant growth promoting rhizobacteria: RAY12, identified as *Achromobacter piechaudii*; RAY28, identified as *Agrobacterium tumefaciens*, RAY132, identified as *Stenotrophomonas maltophilia*; and RAY209, identified as *Delftia acidovorans*. The PGPR act to oxidize elemental sulphur which in turn provides sulphate for the plants. As a result of this arrangement, plants are able to grow more efficiently and effectively and have enhanced growth characteristics, for example (Banerjee and Yesmin, 2002). Hence we can use these bacteria as a bioinoculant for plant growth promotion.

Biocontroling agent

The role of *Thiobacillus* in controlling plant diseases in sulphur amended soils has been demonstrated with regard to potato scab caused by *Streptomyces scabies* and the rot of sweet potatoes caused by *S. ipomoea*.

Under acidic soil conditions (below pH 5.0), inoculation of soil with *thiobacilli* after addition of sulphur effectively minimizes losses due to these pathogens (http://www.western4marketing.com/thiobacillus.php).

Deodorization

Sulphur-oxidizing micro-organisms play a key role in biological deodorization processes. In the prior studies, a number of bacteria capable of oxidizing H_2S were exploited for biological deodorization processes. These bacteria include phototrophic *Chlorobium limicola*, *F. Thiosulfatophilum* (Cork et al., 1986) heterotrophic *Xanthomonas* spp. (Cho et al., 1992) *Pseudomonas putida* (Chung et al., 1996a) and chemoautotrophic *thiobacilli* (Jensen and Webb, 1995). Use of chemo-autotrophic *thiobacilli* for H_2S removal is particularly advantageous because of their simple nutritional requirement, high affinity and removal rate for H_2S and low microbial cell yield (Jensen and Webb, 1995). *Thiobacilli* strains within the species *Acidithiobacillus thiooxidans* (Cho et al., 2000), *Acidithiobacillus ferrooxidans* (Neumann et al., 1990), *Thiobacillus thioparus* (Chung et al., 1996b) and *Thiobacillus denitrificans* (Sublette and Sylvester, 1987) have been reported for their application to H_2S removal in the biological deodorization processes. Removal of H_2S from any effluent, would greatly improve the economics of the process, particularly if this could be achieved micro-biologically. It is therefore of importance to select a microbe that can grow well at ambient temperatures and neutral pH and oxidize sulphide to sulphur in wastewater.

Rubber recycling

Worldwide deposition of waste rubber materials, for example vehicle tyres, constitutes environmental threats and a source of unutilized raw material. The problem with rubber recycling resides in the sulphur cross-links created between the rubber polymers during vulcanization. These cross-links give the material its excellent and characteristic properties but also make it impossible to melt and reshape, as one can do with, for example, glass and plastics. The sulphur-oxidizing bacteria *Acidithiobacillus* and the sulphur-reducing *archaeon Pyrococcus furiosus* have been used to break the sulphur cross-links in vulcanized rubber materials which improved the physical properties of the recycled rubber (Katarina, 2003).

CONCLUSION

The present review highlights the ecology and diversity of

sulphur oxidizing bacteria in different mangrove habitats. Genes and enzyme involved in the sulphur oxidizing process via SOB were exhibited. Finally, the biotechnological potential of such bacteria was treated.

SOB is an important group of microorganisms and widely distributed in all habitats. Though there are much information available on ecology and diversity of SOB, they were only restricted to aerobic enviroemnt. The anaerobic environment like mangroves was sparsely studied by the microbiologists and how SOB sustaining to mangrove ecology is not yet clear. So this review is a collection of recent studies on SOB in different mangrove habitats and their biotechnological application. These microorganisms are not only versatile in their metabolism but also in the environmental conditions in which they thrive. Apart from their importance in nature, SOB, together with sulphur-reducing microorganisms can be successfully exploited in various biotechnological applications such as waste treatment, bioremediation, agriculture, biocontrol etc. Although we have tried to generate information on the diversity and biotechnological application of SOB, we think that we have only scratched the surface and our knowledge of SOB in mangrove ecosystem is still in base line which is one of the greatest challenges in microbial ecology. Therefore, future research is necessary by using innovative technologies to study their ecophysiology, behaviour and interactions with other organisms which will generate enormous opportunities for microbiologists to obtain detailed insights into the diversity, ecology and biotechnology of these important microorganisms.

Conflict of Interests

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

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Review

Resilience of cereal crops to abiotic stress: A review

Mushtaq Ahmad*, Gul Zaffar, S. M. Razvi, Z. A. Dar, S. D. Mir, S. A. Bukhari and Mehfuza Habib

Division of Genetics and Pant Breeding, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir Shalimar campus, Srinagar -191 121, India.

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In the last century, conventional selection and breeding program proved to be highly effective in improving crops against abiotic stresses. Therefore, breeding for abiotic stress tolerance in crop plants should be given high research priority as abiotic stresses are the main factor negatively affecting crop growth and productivity throughout the globe. Advancement in physiology, genetics and molecular biology, have greatly improved our understanding of plant responses to stresses. Many studies show that salt tolerance is tightly associated with the ability to maintain ion homeostasis under salinity. Na^+ transporter SKC1 unloads Na^+ from xylem; plasma membrane N^+/H^+ antiporter SOS_1 excludes sodium out of cytosol, and tonoplast Na^+/H^+ antiporter NHX_1 sequesters Na^+ into the vacuole. Silicon deposition in exodermis and endodermis of rice root reduces sodium transport through the apoplastic pathway. A number of transcription factors regulate stress-inducible gene expression that leads to initiating stress responses and establishing plant stress tolerance. Over expression of some transcription factors, including DREB/CBF and NAC, enhances salt, drought and cold tolerance in rice. A variant of one of ERF family genes, *Sub1A-1*, confers immersion tolerance to lowland rice. These findings and their exploitation will hold promise for engineering breeding to protect crop plants from certain abiotic stresses. Although, cereal crops are also quite sensitive to various abiotic stresses, hence in this short review, we will present recent progresses in adaptation of cereal crops to salinity, drought and cold tolerance are emphasized and the future potentials are highlighted.

Key words: Cereal crops**,** abiotic stresses, food insecurity, molecular breeding, quantitative trait loci (QTLs), salinity, water stress.

INTRODUCTION

Cereal crops assumes its cultivation under much diverse agro-climatic zones extending from truly sub-tropical to cooler temperate regions having altitudes above 7500 ft amsl. Therefore, inevitably, the crop remains open to varied types of biotic as well as abiotic stresses, of which the temperature below 10°C are critical particularly at sowing and maturity in cold temperature areas (Zaffar et al., 2005). Abiotic stresses such as salinity, drought,

*Corresponding author. E-mail: sahilmuhtaqdar@rediffmail.com.

nutrient deficiency or toxicity, and flooding limit crop productivity and such situations become more problematic, where they cause food insecurity for large populations and poverty, particularly in rural areas. For example, drought stress has affected more than 70 million hectares of rice growing land world-wide, while salt stress and nutrient stress render more than 100 million hectares of agricultural land uncultivable thereby resulting in low returns, poverty and reduced educational and employment opportunities. Thus, abiotic stresses are the major factors of poverty for millions of people. Hence, it is widely urged that such strategies be adopted which may be used to get maximum crop stand and economic returns from adverse environments. Major strategies which may be used to overcome the adverse effects of such stressful environments may include development of new crop varieties, screening and selection of the well adapted existing germplasm of potential crops, production of genetically modified (GM) crops, exogenous use of osmo protectants etc. Abiotic stress one among the major causes of crop plant yield losses worldwide. Drought and salinity stress are the major environmental challenges faced by agriculture. Improving yield production and stability of crop plants under stressful environments is important to fulfill food demand of the ever-increasing world population. Though genes associated to plant response(s) to drought and salinity stress have been identified and characterized, in most cases, in the model plant *Arabidopsis*. However, while many of these genes are potential candidates for improving tolerance to abiotic stress, only a small proportion were transferred into crop plants. Further, transgenic crop plants over-expressing the genes of interest were, in most cases, tested under artificial conditions in the laboratory or controlled greenhouse. Thus, while many reports on drought and salinity tolerance in transgenic plants have been published, there is urgent need to test these traits under field conditions. In this chapter, we discuss recent advances in engineering drought and salinity tolerance in crop plants with emphasis on yield and the needs to close the gaps between the laboratory and the field conditions. Crop plants grown under unfavorable environmental conditions prevent the full expression of their genetic potential. The most frequently occurring abiotic stress conditions with adverse effects on crop yield are water, deficit or excess; ions, deficit or excess; temperature, low or high and light, deficit or excess. The ever-increasing human population, concomitant with loss of agricultural land (due to urbanization processes) and diminishing water availability (associated with climate change) pose serious challenges to world agriculture (Mittler and Blumwald, 2010). A significant increase in grain yield of major crop plants such as wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), and maize (*Zea mays* L.) is required to fulfill the food supply requirements for the projected population by 2050 (Godfray et al., 2010). Despite the progress with respect to economic yield already achieved, additional gains in agricultural productivity are demanded at faster pace due to population increase and by changing agricultural practices including biotic and abiotic stresses. Diseases, pests and weed competition losses account for 4.1 and 2.6% yield reductions, respectively, while the remaining reduction (69.1%) is attributed to unfavorable physico-chemical (abiotic) environments induced due to problematic soils and erratic climate patterns. Certainly, some of these losses are caused by inherently unfavorable environments and some by suboptimal management practices by farmers, often due to economic constraints or lack of training. Nevertheless, there is no doubt that a large fraction of potential crop productivity is lost to abiotic stress factors. Plants respond to abiotic stresses at multiple levels such as molecular, cellular, tissue, anatomical, morphological and whole-plant physiological levels (Witcombe et al., 2008) need a through/practical analysis and understanding. The response to stress depends on the duration and severity of the event, as well as the age and developmental stage of the plant, which varies at species and genotype level (Bray, 1997). Typically, early plant establishment (germination and seedling) and the reproductive stage are the most sensitive in determining yield under stress (Zaffar et al., 2005; Barnabas et al., 2008). However, a large segment of the research on abiotic stress credited to *Arabidopsis*, a model system in the past has focused primarily on the vegetative phase identify survival phenotypes, which limits our ability to readily translate the discoveries into improved yield in crop plants.

Abiotic stresses in agriculture are believed to be responsible for reducing crop growth and productivity. Because of their sessile nature, plants at different developmental stages of growth must endure adverse environmental conditions and consequently evolve a variety of responses to acclimatize to environmental stresses. During the course of evolution, plants have developed and acquired sophisticated mechanisms to sense the subtle changes of growth conditions, and trigger signal transduction cascades, which in turn activate stress responsive genes to produce proteins which ultimately lead to changes at the physiological and biochemical levels. A greater understanding of the physiology and molecular biology of stress tolerance may provide a useful platform to breed and develop improved stress-tolerant crop varieties in near future. In modern agriculture, abiotic stresses, especially salinity and drought are major factors limiting crop productivity worldwide. Drought affects plant growth, yield, membrane integrity, pigment content, osmotic adjustments, water relations, and photosynthetic activity (Balachandran et al., 2014). In addition to this, some other factors like low temperatures, heath shock, heavy metals, UV-radiation and pesticides are also affecting crop yields (Bohnert et

al., 1995; Tardieu and Tuberosa, 2010). Abiotic stress is one of the primary causes of crop yield loss worldwide, causing average yield losses of more than 50% in major crops. Tolerance and susceptibility to abiotic stresses are very complex as plant traits that are associated with resistance mechanisms are mutagenic and thus difficult to control and engineer. Drought induces mechanical stress on roots due to soil hardness, osmotic stress because of cell dehydration and removal of water in the extra-cellular space, and oxidative stress by the accumulation of reactive oxygen species (ROS) (Kovtun et al., 2000). Genetic enhancements of cereal crops for improved performance under water-limited environment and high salt is of paramount importance to increase the per capita availability, were such conditions are of frequent occurrence. Developing improved lines for stressful environments requires the rigorous application of molecular breeding and biotechnology. To improve cereal crops further using marker-assisted selection (MAS) and map-based cloning, we require critical information on component traits, accurate phenotyping, the identification of candidate genes and quantitative trait loci (QTLs), the relationship between QTLs and genes, the contribution of individual QTLs to the phenotype, and their variability across different locations and different crop seasons. Another important tool to produce stress-tolerant rice varieties is genetic transformation, which offers a powerful means of incorporating exotic or even synthetic genes with a profound ability to up or down-regulate specific metabolic steps. Rapid progress has been made in developing transformation technologies for field crops and gene transfer need to be done regularly to harness the fruits of this improved technology. Isolation and characterization of novel genes/gene combinations and promoters and their successful transfer into rice will provide new avenues for metabolic engineering for stress tolerance. Such novel genes and promoters offer unique opportunities in genetic engineering of rice for stress environments. Marker aided selection and transgenic approaches are two powerful tools to accelerate plant breeding to produce varieties with improved drought and salt tolerance.

In case of drought, plants improve their water use efficiency either by dehydration avoidance or through dehydration tolerance (Blum, 2005). Due to global warming, it is believed that, India will suffer severe climactic changes in future, including longer drought, lesser amount of total rainfall that is distributed unequally with very heavy precipitation at shorter duration causing flooding, high temperature flux, and higher incidence of tropical storms. Selection among diverse germplasm types will therefore be difficult to meet by following individual characters because of the different response of the genotypes towards improvement for the component to cold tolerance traits. This necessitates the use of some balance selection criteria, which takes into consideration all important cold tolerance attributes simultaneously, so as to end up with overall greater selection advances (Zaffar et al., 2005).

PLANTS RESPONSE TO WATER DEFICIT

Among the various abiotic stress conditions, water deficit is the most devastating factor affecting world agriculture (Araus et al., 2008). About one-third of the world's arable land suffers from chronically inadequate water availability for agriculture, and in virtually all agricultural regions, crop yields are periodically reduced by drought (Bruce et al., 2002). While currently 80% of the world's useable water resources are consumed by irrigated agriculture (Condon et al., 2004) within a few decades, the expanding world population will require more water for domestic, municipal, industrial and environmental needs (Hamdy et al., 2003). This trend is expected to accentuate due to global climatic change and increased aridity (Vorosmarty et al., 2000). Thus, to meet the projected food demands, more crops per drop are required (Condon et al., 2004).

PLANT RESPONSE TO SALINITY STRESS

Salinity is a major constraint on crop-plant productivity (Witcombe et al., 2008). More than 800 million hectares of land throughout the world are salt affected, which accounts for 6% of the world total land area (Munns and Tester, 2008). In most cases, salinity results from natural causes (salt accumulation over long periods of time). In addition, a significant portion of the cultivated agricultural land is becoming saline due to deforestation or excess irrigation and fertilization (Shannon, 1997). Current estimates indicate that 20% of the roughly 230 million hectares of irrigated land is affected by salinity. Given that one third of the food production comes from irrigated agriculture, salinity is becoming a serious problem for crop-plant productivity.

PLANT ADAPTATIONS TO ABIOTIC STRESS

Plant resistance to stress conditions may arise from escape, avoidance or tolerance strategies (Levitt, 1972). Escape relies on successful completion of reproduction before the onset of severe stress (that is, developmental plasticity), achieved by early flowering and/or short growth duration (Mooney et al., 1987). Avoidance involves the prevention or decreasing the impact of the stress on the plant, such as minimizing water loss and maximizing water uptake (Chaves et al., 2003) or exclusion of salt ions, a feature observed in halophytes (Munns and Tester, 2008). Tolerance relies on the inherent

ability of the plant to sustain growth (likely at a reduced rate) even when the conditions are unfavorable for the maintenance of basic plant processes. This strategy involves coordination of physiological and biochemical alterations at the cellular and molecular levels, such as osmotic adjustment (Morgan, 1984) and the sequestration of ion in the plants, in the vacuole or leaf sheath and/or older leaves (Mimura et al., 2003). In most cases, plants subjected to stress conditions combine a suite of responses, exhibiting a number of physiological and biochemical responses at the molecular, cellular and whole-plant level (Chaves et al., 2003). Salinity occurs through natural or human-induced processes that result in the accumulation of dissolved salts in the soil to an extent that inhibits plant growth.

NEW TECHNOLOGIES TO STUDY PLANT RESPONSE TO ABIOTIC STRESS

New technologies are providing opportunities to address the challenging problem of maintaining high-yield crop production under stressful and changing climates. The information provided by high-resolution transcript profiling, the identification of large-scale specific protein networks and their association with the plant responses to environmental perturbations are allowing the application of a systems-level approach to uncover the bases of plant responses to environmental changes. Model plants, such *thaliana* as *Arabidopsis*, *Brachypodium distachyon* and *Medicago truncatula*, have been and will continue to offer insights into the genetic and biochemical basis of abiotic stress adaptations (Bohnert et al., 2006; Hirayama and Shinozaki, 2010). Further, the identification of stress-related genes and pathways has been facilitated by introducing new tools and resources developed in these model plants. Numerous genes related to plant response to stressful conditions of drought and salinity stress have been identified and characterized (Ashraf, 2010; Pardo, 2010). Many of the genes so identified are considered as potential candidates for enhancing tolerance to abiotic stress. In the majority cases, these genes are over expressed in the target plant(s), whether with a strong constitutive promoter or a stress-responsive promoter. Early generations (T1-T3) are screened for responses to stresses to assess the efficacy of the construct. However, majority of these studies were conducted under laboratory conditions (that is, dehydration) in the vegetative phase (that is, seedling, or plate assays) using artificial stress (for example, PEG, mannitol), with very high concentration (that is, osmotic shock) and for short periods (that is, hours). Moreover, most of these studies showed stress tolerance and/or survival, but not the effects of the different stress conditions on plant productivity (Parry et al., 2005). Under rain-fed drought prone agriculture, water stress at the reproductive stage is the most prevalent problem as in most rain-fed ecosystems, the crop season's rains diminish towards flowering and harvest time (Blum, 2009). Thus, more emphasis should be given to the study of the response of crop plants to abiotic stress at the reproductive stage that is under field conditions.

Stress-responsive gene expression

Under abiotic stress (drought, salinity) plants respond either with the activation of synthesis or inhibition of catabolism of osmolytes as sugars (saccharopine, trehalose, raffi nose, galactinol, sorbitol, mannitol, fructans) (Cortina and Culianez-Macia, 2005), sugar alcohols (for example, polyols), amino acids as branched chain aminoacids (BCA), proline and agmatin, quaternary ammonium compounds and small dipeptides as glycinebetaine (Holmstrom et al., 2000). Polyamines (that is, putrescine) are involved in a wide range of plant processes including wounding (Groppa and Benavides, 2008). Besides these functions, such bio-molecules mediate the stress responses. There is a need to develop genotypes having enhanced or faster induction of expression of genes at the crossroad of permissive growth under stress condition. This group of permissive genes includes aquaporin isoforms able to optimize water fluxes (Javot et al., 2003). Several genes have been characterized for their role on stress protection, one among being ERECTA which regulate transpiration efficiency affecting stomatal closure, while the plant is able to maintain biomass production (Masle et al., 2005). Other important regulatory proteins involved in drought stress are proton antiporters as $TNHX_1$ and a proton pyrophosphatase TVP_1 (Brini et al., 2005; Brini et al., 2007a) shown to improve salt and drought stress tolerance in *Arabidopsis*. Dehydrin (accumulate to a high level in the mature embryos) and its isoforms (LEA/DHN) of sizes 17, 30 and 40 kDa, function as intracellular chaperones for other proteins and nucleic acids. One dehydrin (DHN-5) was shown to be involved in salt and drought resistant phenotypes of durum wheat and its accumulation was shown to have a role in preservation of cell integrity during late embryogenesis and desiccation (Brini et al., 2007b).

Dehydrins are also known as late embryogenesis abundant (LEA) or early response to dehydration (ERD) proteins (Beck et al., 2007). It has been shown that LEA/DHNs exert chaperone activity on proteins and nucleic acids (Kovacs et al., 2008; Hara et al., 2009). DHN/LEAs which lack a fixed three-dimensional structure, even though remarkably stable at macromolecular crowding conditions, maintaining a disordered character under conditions (dehydration) in which unfolded states of several globular proteins would tend to collapse (Mouillon et al., 2008). Several LEA/DHN isoforms are

regulated by abscisic acid (ABA) (Jimenez et al., 2008). While the size of induced DHNs was large, ranging between 14 and 74 kDa, drought-tolerant Bermuda grass varieties showed to induce preferentially the 31 and 40 kDa isoforms (Hu et al., 2010). Roots grow and recover their function after environmental stresses through specific genes that adapt root development to these restrictive conditions. The recovery of *Medicago truncatula* roots after a salt stress is mediated by regulatory networks depending on TFIIIA-like transcription factors, involved in the control of root adaptation to salt stress. Those conditions induced synthesis of a novel RNA-binding protein, a small G-protein homologous to ROP_9, a receptor-like kinase, two TF IIIA-like and an AP2-like transcription factors (TF), MtZpt2-1, MtZpt2-2 and MtAp2, and a histidine kinase associated with cytokinin transduction pathways (Merchan et al., 2007). A full-length 1.1 kb cDNA, designated *O. sativa* Dehydrin 1 (OsDhn1), was isolated from the seed coat of rice. The deduced protein is hydrophilic and has three K-type and one S-type motifs (SK*3*-type), indicating that OsDhn1 belongs to the acidic dehydrin family, which includes wheat WCOR410 and Arabidopsis COR47. Expression of OsDhn1 was strongly induced by low temperature as well as drought. Its induction by cold stress was clear cut in the roots of seedlings and the epidermis of palea and lemma, while it was also up-regulated in UBI: CBF1/DREB1b transgenic plants indicating that it is regulated by the CBF/DREB stress signaling pathway.

In spite of a surge in literature on drought tolerance in crops during the past two decades, practical progress in breeding for drought tolerance has not been significant. Drought therefore requires an analytical approach of dissecting and studying the contribution of trait components using the quantitative trait loci (QTLs) model. This approach is particularly suited to crops like rice for which dense genetic linkage maps with a variety of DNA markers are already available. Most studies on drought tolerance deal with evaluating specific traits logically related to crop performance under drought. A molecular genetic analysis is more effective if conducted on individual traits (and even their components) before crop performance is considered. Thus, it is important to phenotype for specific traits or responses under managed nursery or greenhouse conditions, besides subsequent field evaluations under drought in target regions. A strong demonstrated linkage between traits in relation to crop performance in the target environment is a crucial step before advocating marker-assisted selection (MAS). A second approach is to create a novel and functionally known type of variability in plant stress response by genetic transformation.

The transgenic approach offers a powerful means of incorporating a broad spectrum of genes with profound ability to regulate specific metabolic paths associated with stress response. Although transformation with any single gene or group of genes for a particular pathway may not be adequate for conferring drought tolerance as in majority of cases several pathways are required to be carried out and the outcome of these paths (products) are required to ensure drought tolerance (Ingram and Bartels, 1996). In general, many proteins and low-molecular-weight osmolytes accumulate under stress. It is not clear which factor(s) among many changes contributes to the development of stress tolerance because so many changes occur. Thus, transformation approach is useful, to obtain valuable information. By transferring a single gene into a plant, and then studying the response of the resulting plants to drought or salt stress, one can clearly understand whether or not a given gene has a well defined role in controlling a particular stress condition, and what related changes occur. This approach will thus be useful in identifying candidate genes for stress tolerance or its components with significant developments in gene transfer technologies for rice and rapid progress in gene isolation and manipulation.

Signalling pathway

Emergence of the novel “omics” technologies, such as genomics, proteomics, and metabolomics, allows researchers to identify the genetic bases behind plant stress responses. These technologies enable a direct and unbiased monitoring of the factors affecting plant growth and development and provide the data that can be directly used to investigate the complex interplay between the plant, its metabolism, and also the stress caused by the environment or the biological threats (insects, fungi, or other pathogens). Plant responses to stress are mediated via profound changes in gene expression which result in changes in composition of plant transcriptome, proteome, and metabolome (Alfocea et al., 2011).

Genomics

Functional genomics allows large-scale gene function analysis with high throughput technology and incorporates interaction of gene products at cellular and organism level. The information coming from sequencing programs is providing enormous input about genes to be analyzed.

The availability of many plant genomes nowadays (Feuillet et al., 2010) facilitates studying the function of genes on a genome wide scale. The lack of information from other plant genomes will also be compensated in part by the availability of large collection of expressed sequence tags (ESTs) and cDNA sequences. The basic interest behind these EST projects is to identify genes responsible for critical functions.

ESTs, cDNA libraries, microarray, and serial analysis of gene expression (SAGE) are used to analyze global gene expression profiles in a functional genomics program. Large mutant collections are tools that complement large-scale expression studies. Gene identification through physical and chemical mutagens has become amenable for large-scale analysis with the availability of markers (Lukowitz et al., 2000), but gene tagging is more promising for functional analysis on a wider scale. Moreover, the understanding of the complexity of stress signaling and plant adaptive processes would require the analysis of the function of numerous genes involved in stress response. Numerous investigations show that plant defense response genes are transcriptionally activated by pathogens and also by different types of abiotic stress. It has been described that the induction of specific defense genes, in the response against certain pathogens, is dependent on specific environmental conditions, suggesting the existence of a complex signaling network that allows the plant to recognize and protect itself against pathogens and environmental stress. Similar induction patterns of members of the 14.3.3 gene family (GF14b and GF14c) by abiotic and biotic stresses such as salinity, drought, ABA, and fungal inoculation have been documented in rice (Chen et al., 2006). The rice GF14 genes contain cis elements in their promoter regions that are responsive to abiotic stress and pathogen attack. The 14-3-3s family genes are also subject to the regulation by certain transcript factors. On the other hand, kinase cascades of the mitogen-activated protein kinase (MAPK) class play a remarkably important role in plant signaling of a variety of abiotic and biotic stresses, and it is an essential step in the establishment of resistance to pathogens (Pitzschke et al., 2009). It has been described that in Arabidopsis, MEKK1 and ANP1 act in the environmental stress response (Suarez-Rodriguez et al., 2007), and MPK3, MPK4, and MPK6, are activated by a diversity of stimuli including abiotic stresses, pathogens, and oxidative stress (Qiu et al., 2008). Elucidating the molecular mechanism that mediates the complex stress responses in plants system is an important step to develop improved variety of stress tolerant crops. Many crop traits are quantitative, complex, and controlled by multiple interacting genes. Recent progress in molecular biology provides the tools to study the genetically make-up of plants, which allows us to unravel the inheritance of all traits whether they are controlled by single genes or many genes acting together, known as the quantitative trait loci (QTL). The molecular marker technologies available since the 1980s allow dissecting the variation in traits. With the progress of QTL mapping, new breeding approaches such as marker assisted selection and breeding by design have emerged (Peleman and Voort, 2003). Advances in plant genomics research have opened up new perspectives and opportunities for improving crop plants and their productivity.

The genomics technologies have been found useful in deciphering the multigenicity of biotic and abiotic plant stress responses through genome sequences, stress-specific cell and tissue transcript collections, protein and metabolite profiles and their dynamic changes, protein interactions, and mutant screens.

Proteomics

The adaptation of plants to biotic or abiotic stress conditions is mediated through deep changes in gene expression which result in changes in composition of plant transcriptome, proteome, and metabolome. Since proteins are directly involved in plant stress response, proteomics studies can significantly contribute to elucidate the possible relationships between protein abundance and plant stress acclimation. Several studies (Bogeat-Triboulot et al., 2007) have already proven that the changes in gene expression at transcript level do not often correspond with the changes at protein level. The investigation of changes in plant proteome is highly important since proteins, unlike transcripts, are direct effectors of plant stress response. Proteins not only include enzymes catalyzing changes in metabolite levels, but also include components of transcription and translation machinery.

Metabolomics

The possibility of monitoring a complete set of metabolites could largely improve the understanding of many physiological plant processes. This systematic study, defined as “metabolomics,” is intended to provide an integrated view of the functional status of an organism. Besides, its use as a breeding or selection tool, metabolomics techniques have also been used to evaluate stress responses in barley (Widodo et al., 2009), Citrus (Djoukeng et al., 2008), *M. truncatula* (Broeckling et al., 2005), and *Arabidopsis thaliana* (Fukushima et al., 2011).

ROLE OF JASMONIC ACID (JA) AND ABSCISIC ACID (ABA) IN STRESS SIGNALLING

Plant hormones play important role in all the developmental stages of plant, as they regulate all the important functions from the germination till the death of any particular plant/part. These bio-molecules regulate the commitment of plants to growth or senescence under abiotic stress. The plant copes with a specific stress through complex signals circulating from roots to shoots and leaves and delivering hormones and signalling back to the roots, activating the expression of protective proteins

or down-regulating unnecessary pathways.

These compounds sustain signals at short distance, between cells, or in a systemic way in the case of transport systems. In this review, we will try to highlight the role exerted by jasmonic acid (JA) and abscisic acid (ABA), as the former is the final product of the octodecanoic pathway which uses linoleic acid as substrate to produce oxylipins (Hughes et al., 2009). The octodecanoic pathway starts with the oxygenation of a polyunsaturated fatty acid (PUFA) by lipoxygenase (LOX), to form a fatty acid hydroperoxide. In plants, only the type-2 13-LOXs are believed to be associated with JA biosynthesis (De Domenico et al., 2007). In the biosynthesis of JA, the subsequent activities of 13-LOX, allene oxide synthase (AOS), allene oxide cyclase (AOC) lead to formation of cis- (+)-12-oxophytodienoic acid (OPDA), a JA precursor. JA is methylated by a specific JA-methyltransferase, which forms a volatile compound able to cross plasma membranes and exert its action at distance. Another effector in the lipoxygenase biosynthesis pathway is jasmonate-conjugated isoleucin (JAIle) that may accumulate stored in organelles and vacuoles. JA-Ile is able to translocate through membranes and move through xylem from roots to leaves and backward. Once the hormone has arrived at its destination in the target cell (locally or at distance), the priming of jasmonate-inducible genes is regulated by convergent pathways, linked to phosphoinositide and ABA-dependent signalling components.

ROLE OF ABSCISIC ACID IN ABIOTIC STRESSES

Abscisic acid (ABA) is a growth regulator involved in senescence, seed dormancy, plant development, drought tolerance and stress response, synthesized in the xylem and in the aerial parts of the plant, where it regulates stomatal movement and the activity of shoot meristems. ABA can flow in the root cortex across apoplastic barriers and play an important role in the regulation of signal intensity. The abscisic acid glucose ester (ABAGE) is a long-distance stress signal, stored in microsomes, and released by activated betaglucosidases, both in the apoplast and the cytosolof the mesophyll cells. ABA-GE transporters located on plasma membrane of the xylem parenchyma cells influence ABA mobility. Two intense sources of ABA absorption include internal and external sources. External ABA originates from root exudation and from ABA-producing soil organisms (predominantly fungi), whereas internal ABA comes from its biosynthesis at root shoots level and phloem import (Sauter and Hartung, 2000; Sauter et al., 2001). It has been reported that conjugated ABA (ABA-GE) also occurs in the soil, often in higher concentrations than ABA. The Casparian bands of the exodermis and endodermis are perfect barriers for ABA-GE. When an exodermis is absent (*Fabaceae* and hydroponically cultivated plants), external ABA-GE enters into the apoplast of the root cortex. Apoplastic β-glucosidases can cleave the conjugated form and release free ABA, which is distributed to the symplast and/or transported across the endodermis into the xylem (Hartung et al., 2002).

SALT STRESS AND PHOSPHATE DEFICIENCY

Strong ABA synthesis and accumulation in the roots can be observed in plants affected by hemi parasites such as *Rhinanthus minor* (Jiang et al., 2004). ABA biosynthesis in the roots was reported to be 12-fold higher after attack, resulting in 14-fold higher ABA flows in the xylem. Plants regulate inorganic phosphate (Pi) homeostasis to adapt to environmental changes in Pi availability. Some degree of cross-talk between ABA and other signalling pathways was reported in phosphate limitation conditions. This mechanism involves phosphate uptake increase from the soil and phosphate mobilization from the leaf. Upon Pi starvation, up regulated miR399 cleaves its target gene, PHO_2, in *A. thaliana*, an ubiquitin conjugating E_2 enzyme, thereby releasing several protein targets from ubiquitin-pathway dependent degradation and increasing Pi content in the shoots (Franco-Zorrilla et al., 2007).

TRANSCRIPTION FACTORS AND ABIOTIC STRESSES

Transcriptional regulation, also known as transcriptome reprogramming, is essential for plant adaptation to abiotic stresses. Till date, multiple transcription factors required for transcriptome reprogramming under abiotic stresses have been identified and functionally analyzed for several crop species. Among them, some have been well addressed in rice, for example, DREBs (dehydration responsive element-binding protein) /CBFs (C-repeat-binding factor) and NACs (NAM, ATAF, and CUC). These transcriptome reprogramming in rice needs to be utilized to improve adaptation of stress responsive traits in rice.

DREBs/CBFs

The DRE (dehydration-responsive element)/CRT (C-Repeat) were identified as a *cis*-acting element regulating gene expression in response to dehydration (salt, drought, and cold stresses) in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki, 1994). Transcription factors DREB1/CBF1-3, CBF4 and DREB2, belonging to the ERF/AP2 family, were reported to bind to DRE/ CRT elements (Yamaguchi-Shinozaki and Shinozaki, 2005). While three *DREB1/CBF1-3* genes, *DREB1A/CBF3, DREB1B/CBF1* and *DREB1C/CBF2,* were induced by cold, but not by drought or salt stress (Medina et al., 1999), and were consequently believed to regulate the

expression of DRE/CRT genes under cold, *DREB2A* and *CBF4* were mainly responsive to drought and salt stresses (Haake et al., 2002). Interestingly, over expression of *DREB1s* in *Arabidopsis* increased freezing, drought, and salt tolerance, however, over expression of *DREB2A* in transgenic plants showed no increase in stress tolerance (Liu et al., 1998). These data suggest activation of DREB2A requires post-translational modification. Sakuma et al. (2006) found that the deletion of a region between residues 136 and 165 transformed DREB2A to a constitutively active form. Transgenic *Arabidopsis* plants expressing this active form exhibited improved drought tolerance, and slight freezing tolerance as well. It suggested the region between residues 136 and 165 of DREB2A is an inhibitory domain in the normal condition and is modified under salt/drought stress.

The functions of DREB orthologs have been demonstrated in rice, and five DREB cDNAs identified: *OsDREB1A*, *OsDREB1B*, *OsDREB1C*, *OsDREB1D* and *OsDREB2A*. Similar to their homologs in *Arabidopsis*, *OsDREB1A* and *OsDREB1B* were induced by cold; while *OsDREB2A* was regulated by salt and drought stress (Dubouzet et al., 2003). However, rice DREBs binding sites differed from their AtDREB counterparts as OsDREB1A showed much higher affinity binding to the DRE core sequence with GCCGAC than that with ACCGAC (Dubouzet et al., 2003), while AtDREBs bound equally to both sequences (Stockinger et al., 1997; Liu et al., 1998). Overexpression of OsDREB1A in *Arabidopsis* and rice induced expression of DRE/CRT genes (Dubouzet et al., 2003; Ito et al., 2006) and OsDREB1A overexpression lines showed phenotypes similar to AtDREB1A over expression lines, with improved stress tolerance as well as growth retardation under normal growth conditions (Dubouzet et al., 2003; Ito et al., 2006). These results demonstrate that the DREB1/CBF pathway was conserved in monocotyledons and dicotyle-dons. Contrary to the growth inhibition observed in cv. Kita-ake and Nipponbare rice, overexpression of *Arabidopsis* DREB1A in rice cv. Nakdong enhanced tolerance to abiotic stress without inhibiting growth or causing phenoltypic alterations (Oh et al., 2005). The discrepancies observed between the different studies may have resulted from genotype differences, as observed by comparative analysis of their transcriptomes (Ito et al., 2006).

NAC genes

The *NAC* gene family encodes one of the largest families of plant-specific transcription factors and has not been found in other eukaryotes. There are 75 and 105 putative *NAC* genes in rice and *Arabidopsis* genomes, respectively (Ooka et al., 2003). Genes in the *NAC* family were found mainly to be involved in regulating plant development (Olsen et al., 2005). Their roles in abiotic stresses were only discovered recently. A salt- and drought-induced gene, *ERD1*, was regulated in an ABA-independent manner (Kiyosue et al., 1993; Nakashima et al., 1997). However, no DRE/CRT element was found in its promoter region, suggesting a novel regulatory pathway for drought and salt adaptation (Kiyosue et al., 1993; Nakashima et al., 1997). Promoter analysis showed that an MYC-like site was necessary for induction of *ERD1* (Simpson et al., 2003). The MYC-like sequence was recognized by three transcription factors of the NAC family, ANAC019, ANAC055, ANAC072, and was named NACRS (NAC recognition sequence) (Tran et al., 2004). Consistent with *ERD1* expression patterns, the three *NAC* genes were induced under salt and/or drought stress, but were not remarkably regulated by cold (Tran et al., 2004). In addition to *ERD1*, many other salt and/or drought stress-induced genes were also regulated by the three genes, and consistently, over expression of these genes greatly enhanced drought tolerance in model plant *Arabidopsis* (Tran et al., 2004). In rice a similar set of NAC transcription factors may be used to regulation salt and/or drought responsive genes. In this direction, Chao et al. (2005) reported that multiple rice transcription factors, including a *NAC* gene, were induced in the early stage of salt stress. OsNAC6, a member of ATAF subfamily, was also induced by cold, salt, drought and abscisic acid (ABA) (Ohnishi et al., 2005). However, the precise functions of these NAC genes remain largely unknown, while recently (Hu et al., 2006) reported a NAC transcription factor significantly enhanced drought and salt tolerance in rice. The rice NAC gene *SNAC1* was up-regulated by drought and salt predominantly in guard cells. *SNAC1*- overexpressing plants showed greater sensitivity to ABA and increased stomatal closure to prevent water loss in rice. Drought resistance in transgenic plants was significantly improved under field conditions at the stage of anthesis, without phenotypic changes or yield reduction. However, although SNAC also triggered a series of salt and/or drought responsive genes including *OsERD1*, differences were noted in the regulation controlled by SNAC compared to ANACs, as the former could not interact with NACRS in the *OsERD1* promoter region. These data, in conjunction with differences noted between DREB/CBF regulons in rice and *Arabidopsis*, suggested that stress-related regulation pathways further evolved after the divergence of monocotyledons and dicots. Hence a thorough analysis and understanding in these systems is the need of hour to develop genotypes either with molecular or transgenic approaches to reach a productivity level, which can meet the dependant/increasing population on rice as stable food.

Other transcription factors

Although multiple transcription factors, including ICE

(inducer of CBF expression), CBFs/DREBs, AREB/ABF/ABI/bZip, MYC/ MYB and NACs, have been well characterized (Chinnusamy et al., 2004; 2006), we are far from fully understanding transcriptional reprogramming under salt/drought stress. It was estimated that about 8% of yeast genes were affected by salt stress (Zhu, 2002). If a similar percentage was assumed for rice, there would be about 4000 genes responsive to salt stress. To date, hundreds of salt responsive genes have been identified in cereal crops using high throughput technologies, such as microarray/gene chip (Chao et al., 2005; Wu et al., 2006). Although, these numbers are small compared with the potential 4000 genes, they cannot be fully explained by previously identified regulatory pathways. In *Arabidopsis*, a comparison of a transcriptome under cold and a CBF regulon revealed that only 12% of cold responsive genes were regulated by CBFs (Fowler and Thomashow, 2002). In addition, even in the CBF regulon, a few of the genes did not display DRE/CRT elements in their promoter region. It has consequently been hypothesized that subregulons control those genes without a DRE/CRT element, given that some transcription factors with DRE/CRTs in their promoters, for example *RAP2.1*, were represented in the CBF regulon (Fowler and Thomashow, 2002). A designed microarray has been used to analyze the response of transcription factors to biotic and abiotic stress, and demonstrated that more than 28 transcription factors were induced by abiotic stress (Chen et al., 2002). In rice, many transcription factors, including zinc finger, NAC, bHLH, MYB and WRKY, has also been reported to be induced by salt and drought stresses. Extensive research (Wu et al., 2006) also identified multiple transcription factors that were induced by stress and interestingly Chao et al. (2005) reported that transcription factors were rich in the earliest salt induced genes. These data suggest that multiple regulatory pathways under salt/drought stress remain to be characterized.

STRATEGIES FOR IMPROVING CROPS, AGAINST WATER AND SALT STRESSES

As mentioned earlier, both water stress reduces plant growth and crop productivity, so it is imperative to reduce yield gaps by increasing crop drought tolerance under these conditions, thereby ensuring food security for the increasing human population as well as for the benefit of poor farmers world-over. In this context, crop stress tolerance is defined in terms of yield stability under abiotic stress conditions. However, yield losses caused by abiotic stresses vary depending on timing, intensity and duration of the water stress, coupled with other environmental factors such as high light intensity and temperature. Based on this information, following means are suggested (Parry et al., 2005; Neumann, 2008) to reduce/overcome the losses caused due to such stresses:

(i) Water management practices that save irrigation water.
(ii) Exploitation of the agronomic practices by which plants can perform well under water stress conditions.
(iii) Selection of crop cultivars that require relatively lower quantity of water for their growth and crop productivity.

Strategies involving water saving irrigation technologies or cultural practices to alleviate drought stress, are expensive, inconvenient, and require specific knowledge for its implementation. On the other hand, use of drought resistant crop plants in drought prone environment, that is, biological approach is more feasible and efficient in achieving high crop productivity on drought hit areas. In addition, the biological approach involves, those methodologies which are used to enable plants that can effectively escape, avoid or tolerate drought.

SELECTION AND BREEDING FOR DROUGHT TOLERANCE

The development of drought-resistant cultivars/genotypes for any particular crop through conventional breeding method has been and still is of considerable economic value for increasing crop production in areas with low precipitation or with improper irrigation system (Subbarao et al., 2005). However, availability of genetic variation at inter-specific, intra-specific and intra-varietal levels is of prime importance for selection and breeding for enhanced resistance to any stress (Serraj et al., 2005). In order to develop drought resistant/ tolerant cultivars, it is imperative to develop efficient screening method and suitable phenotyping criteria. Various agronomic, physiological and biochemical selection methods for drought tolerance are being employed to select drought tolerant plants, such as seed yield, harvest index, shoot fresh and dry weight, leaf water potential, osmotic adjustment, accumulation of compatible solutes, water use efficiency, stomatal conductance, chlorophyll fluorescence (Neumann, 2008). Development of drought tolerance in adaptation for a plant is the result of overall expression of many traits in a testing environment. Since many adaptative traits are effective only for certain aspects of drought tolerance and over a limited range of drought stress, there is no single trait that breeders can use to improve productivity of a given crop in a water deficit environment. Therefore, alternative potential systematic approach is to pyramid various traits in one plant genotype which can improve its drought tolerance. In this context, Subbarao et al. (2005) suggested that those traits, whether physiological or morphological, that contribute to reduce water loss through transpiration, and enhance water use

efficiency and/yield are traits of interest. While discussing prospects for crop production under drought, Parry et al. (2005) suggested some key traits to be keeping in consideration while breeding for drought tolerance (for example, phenology, rapid establishment, early vigor, root density and depths, low and high temperature tolerance, ^{13}C discrimination (a measure of the extent to which photosynthesis is maintained while stomatal conductance decreases), root conductance, osmoregulation, low stomatal conductance, leaf posture, habit, reflectance and duration, and sugar accumulation in stems to support later growth of yield components). However, they stressed that priority should be given to those traits that will maintain or increase yield stability in addition to overall yield, because traits for higher yield may in fact decrease yield stability (longer growth period). Thus, in order to improve crop productivity under water stress conditions, selection of a cultivar with short life span (drought escape), incorporation of traits responsible for well-developed root system, high stomatal resistance, high water use efficiency (drought avoidance), and traits responsible for increasing and stabilizing yield during water stress period (drought tolerance) should be given high priorities. Although a number of crop cultivars tolerant to drought stress have been developed through this method, this approach has been partly successful because it requires large investments in land, labor and capital to screen a large number of progenies, and variability in stress occurrence in the target environment. In addition, there is an evidence of marginal returns from conventional breeding, suggesting a need to seek more efficient methods for genetic enhancement of drought tolerance.

Molecular breeding

Now, it is well evident that water stress tolerant traits are mainly quantitative in nature and are controlled by multiple genes or gene complexes. The regions of chromosomes or the loci controlling these traits are called quantitative trait loci (QTLs). In QTL approach of plant breeding, parents showing extreme phenotypes for a trait are crossed to produce progenies with a capacity of segregation for that trait. This population is then screened for genetic polymorphism using molecular markers technique such as RFLP, RAPD, AFLP and SNPs. Genetic maps are being constructed and markers associated with a trait of interest are identified using computer software. Use of molecular markers to identify QTLs for physiological traits responsible for stress tolerance has helped to identify some potential sub-traits for drought tolerance (Hussain, 2006). Once molecular markers (that is, for a target QTLs) are linked to specific sub-traits of drought tolerance, it would be possible to transfer these various traits into other adapted cultivars with various agronomic backgrounds under specific targeted environments through marker assisted breeding approaches.

Thus, identification of areas of a genome that have a major influence on drought tolerance or QTLs for drought tolerance traits could allow to identify the genes for drought tolerance. Thus, use of marker-assisted selection (MAS) seems to be a more promising approach because it enabled us to dissect quantitative traits into their single genetic components thereby helping in selecting and breeding plants that are resistant to any target trait like water stress (Chinnusamy et al., 2004; Hussain, 2006). The identification of QTLs for economically important traits has been achieved by developing linkage mapping to anonymous markers (segregation mapping) or through association studies (association mapping or candidate gene approach) involving candidate genes (Araus et al., 2003).

Although, most of the data for QTLs for drought tolerance available in the literature is based on segregation mapping studies (Cattivelli et al., 2008), association mapping or candidate gene approach is more vigorous than segregation mapping (Syvänen, 2005), because single genes controlling a trait such as flowering time, plant height, ear development and osmotic adjustment may have more important role in adaptation to drought-prone environment, as single candidate gene (*or* gene) conferring osmotic adjustment in wheat was mapped on the short arm of chromosome 7A (Morgan and Tan, 1996) and breeding for *or* gene improved yield in wheat under water deficit conditions (Morgan, 2000). While critically analyzed the reports on the application of QTL analysis (Cattivelli et al., 2008) pointed out that more efforts have been dedicated to understand the genetic basis of physiological traits responsible for drought tolerance, and little attention has been given to understand high yield stability in water deficit conditions. For example, more reports are available on genetic variation for osmotic adjustment, genetic basis of phonological traits, the ability of roots to exploit deep soil moisture, water use efficiency, limitation of non-stomatal water loss, and leaf elongation rate under varying degrees of water stress. Detailed information on QTLs for drought tolerance is available as GRAMENE (http://www.gramene.org/) or GRAINGENES. However, despite theoretical advantages of utilizing MAS to improve quantitative traits during the past decade, the overall impact of MAS on the direct release of drought-tolerant cultivars remains non-significant (Reynolds and Tuberosa, 2008). In view of the available information, identification of QTLs responsible for improving yield potential and drought tolerance will be main goal for the present and future research. Thus, it is suggested that deliberate selection for secondary traits related to drought tolerance is likely to achieve better results than direct selection for yield *per se* under stress conditions (Tuberosa et al., 2007). Marker assisted selection will

become more efficient if available markers are tightly linked to the genes of interest like stress related traits. Interestingly, Babu et al. (2003) while working with rice, found that QTLs for plant yield under drought were coincided with QTLs for root traits and osmotic adjustment. Likewise, Lanceras et al. (2004) found that favorable alleles for yield components were located in a region of rice chromosome 1 where QTLs for many drought related traits (root dry weight, relative water content, leaf rolling and leaf drying) were previously identified. However, in this strategy, parents of extreme contrasting traits (yield and drought tolerance) are required which may cause a cost on grain yield by decreasing yield component traits. From all this discussion, it seems that with the advent of this high throughput molecular biology technique, we are probably on the threshold of breakthroughs in our ability to understand and manipulate plant physiological responses to water deficit. Although use of molecular marker-assisted selection (MAS) seems to be more promising and meaningful, the contribution of molecular breeding to the development of drought tolerant cultivars has so far been marginal and a few reports are available in this regard (Cattivelli et al., 2008).

Another important application of molecular breeding is cloning of genes/DNA sequences associated with QTLs for drought tolerance. A number of strategies are being used to clone candidate genes/DNA sequences (Salvi and Tuberosa, 2005), which are evident from the available literature, by mapping of known stress responsive genes (Tondelli et al., 2006). For example, (Masle et al., 2005) cloned ERECTA gene in *A. thaliana*, a DNA sequence beyond a QTL for transpiration efficiency. However, there is no report available in the literature on cloning of genes underlying QTLs in any crop species. For identification of QTL corresponding gene (QTN-quantitative trait nucleotide), generation of molecular-linkage maps based on candidate genes (molecular function maps) is suggested to avoid time consuming fine mapping by a number of researchers. For example, this strategy has been applied to find genes for drought tolerance in barley and rice (Tondelli et al., 2006).

Molecular biology approaches to increase crop salt tolerance

Although, salt tolerance in plants is determined by a number of physiological and biochemical traits, but it is now well evident that salt tolerance is a complex trait involving the function of many genes (Munns, 2005; Munns and Tester, 2008). Furthermore, successful screening and selection of salt tolerant cultivars in conventional breeding program is limited by the significant influence of environmental factors affecting the expression of this polygene's (Ashraf et al., 2008). In view of this argument, it is suggested to identify the molecular markers tightly linked to the genes governing salt tolerance which could indirectly be used to select plants in segregating populations as molecular markers are environment independent. Thus, the use of QTLs has improved the efficiency of selection, particularly, for those traits that are controlled by many genes and are highly influenced by environmental factors (Flowers, 2004).

Salt tolerance in plants varies with the change in growth stage that cause problem in selecting salt tolerant genotypes. Although, QTLs for salinity tolerance have been identified in a number of potential cereal crops such as rice, barley and wheat, robust markers that can be used across a range of germplasm are limited (Munns, 2008). Since 1993, a number of reports are available in the literature showing enhanced salt tolerance in different crop plants by over-expressing genes that are involved in controlling traits responsible for salt tolerance (Flowers, 2004; Munns, 2005; Ashraf et al., 2008).

Munns (2005) categorized these salt tolerant genes into three different categories (1) those that control salt uptake and transport; (2) that have an osmotic or protective function; and (3) that could make a plant grow more quickly in saline soil. However, large numbers of successful reports from transformation experiments have come from manipulating genes responsible for Na^+ exclusion or tissue Na^+ tolerance (Munns and Tester, 2008). These claims of improved salt tolerance were highly criticized because of poor experimental designs, inappropriate choices of methods to evaluate for salt tolerance (Flowers, 2004; Munns, 2005; Ashraf et al., 2008).

Which trait is useful, and which parent contributes?

One of the interesting revelations of the QTL analysis is that both parents (drought tolerant and -sensitive) contribute useful alleles for the trait of interest. Traditional grouping of drought-tolerance traits into four distinct classes (phenological, morphological, physiological and biochemical) may still be relevant for identifying QTLs. Because phenological and morphological traits can be scored easily in appropriately managed screening nurseries, the QTLs identified for these traits may be more reliable. Physiological traits such as osmotic adjustment or water-use efficiency are cumbersome to measure in the field, and can be relied on only under a carefully defined set of conditions. Biochemical traits, on the other hand, defy simple characterization, and most often they may only be the symptoms of stress. But they will have an increasing role in increasing our understanding of drought-tolerance mechanisms. The association between biochemical traits and QTLs is too speculative at the moment.

CONCLUSION AND FUTURE PROSPECTS

Although, it is widely recognized that salt and drought stresses are major constraints for crop productivity, knowledge about nature and magnitude of both stresses is scanty to develop an economically viable/sustainable agriculture. For example, a great gap exists in knowledge about the level of stress tolerance to be developed in crops intended to be grown on a targeted environment. Such kind of knowledge will certainly be helpful in prioritizing traits/selection criteria and developing screening techniques for improved stress tolerance. During the last two decades, plant breeders have been able to successfully develop cultivars with at least some tolerance for a number of abiotic stresses by exploiting genetic variation that exists among the cultivated varieties. Inter- and intra-specific genetic variation for stress tolerance in the present germplasm has resulted from long-term farmer selection or from wild relatives of crop plants that have evolved abiotic stress tolerance as a means to allow colonization of marginal and extreme habitats. However, desired diversity for improving stress tolerance is not available though small increase in stress tolerance feasible by exploiting existing genetic variation. In order to increase the extent of existing genetic variation for stress tolerance, use of wide hybridization, molecular breeding or transgenic approaches are suggested. Although wide hybridization can enhance the stress tolerance, it may cause a significant penalty in terms of yield. Development of transgenic plants for transcription factors, antiporter and compatible solutes resulted in enhanced stress tolerance in plants. However, such types of reports on enhanced stress tolerance are highly criticized due to adoption of poor evaluation methodology in carrying out such studies. At present, we are still unaware about stress-induced changes in metabolism in plants, and a major gap in our understanding of stress tolerance. With the advancement in functional genomics, it is possible to identify key genes and their immediate functions at cellular as well as at whole plant level. Thus, detailed analysis of underlying physiological and molecular mechanisms for salt tolerance using functional genomics is an important area of future research, which will eventually assist in developing transgenic plants for stress tolerance. Therefore, the improvement in abiotic stress tolerance in agricultural plants can only be achieved practically by combining traditional and molecular breeding approaches. In the meantime, it would be sensible to use shotgun approaches (exogenous application of compatible solutes, plant growth regulators, antioxidant compounds, inorganic salts) to increase salt tolerance in potential crops. Moreover, genetic modification should be combined with marker-assisted breeding programs with stress-related genes and QTLs, and ultimately, the different strategies should be integrated, and genes representing distinctive approaches should be combined to substantially increase plant stress tolerance. Through more widespread application of forward and reverse genetic analyses in model plants and with the growing power of genomics and proteomics tools, progress in understanding abiotic stress signaling will certainly accelerate. With a better understanding comes more effective ways to improve plant tolerance to abiotic stress. A new world in modern agriculture is coming nearer and nearer.

Conflict of Interest

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

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Immunodiagnostic potential of a 27 kDa protein of *Fusarium xylarioides,* the cause of coffee wilt disease in Robusta coffee in Uganda

Olal, S.[1]*, Atuhaire, K. D.[2,4*], Ochwo, S.[4], Kiiza, L.[1], Kangire, A.[1], Musoli, P.[1], Olaho-Mukani, W.[3], Lubega, G. W.[4] and Hakiza, G. J.[1]

[1]National Agricultural Research Organization, National Coffee Resources Research Institute, P.O.BOX 185 Mukono, Uganda.
[2]National Agricultural Research Organization, National Livestock Resources Research Institute, P.O.BOX 96, Tororo, Uganda.
[3]African Union-Interafrican Bureau of Animal Resources, P.O. BOX 30786, Nairobi, Kenya.
[4]College of Veterinary medicine, Animal resources and Biosecurity, Makerere University, Kampala, Uganda.

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Several *Fusarium* species infect Robusta coffee; these *Fusarium xylarioides* Steyaert (*Gibberella xylarioides* Heim and Saccas) are the most virulent and responsible for the destructive Robusta coffee wilt disease in Uganda. To date, *F. xylarioides* has not been isolated directly from soil, though the pathogen can persist in soil for a short time. In this study, a promising diagnostic target which can be developed into a serological test for *F. xylarioides* in coffee plants and soil has been identified and validated for identification. Water-soluble extracts of mycelia from six *Fusaruim* species were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The different protein profiles from the other five *Fusarium* species were compared and contrasted with that of *F. xylarioides*. Protein bands that appeared peculiar to *F. xylarioides* were cut and injected into rabbits to produce polyclonal antibodies. Dot blot and Western blot analyses showed one immunodominant antigen (27 kDa) common to all *F. xylarioides* isolates analyzed. No cross-reactivity of anti-27 kDa antibodies were observed in the entire test *Fusarium* species. The results suggest that polyclonal antibodies raised against the endoantigens from *F. xylarioides* of 27 kDa, is a promising tool for the rapid, sensitive, and accurate detection of pathogen in soil and plant parts.

Key words: *Gibberella xylarioides*, coffee wilt disease, antigen, antibodies, Uganda.

INTRODUCTION

One of the major constraints of Robusta coffee (*Coffea canephora* Pierre) production in Uganda is the coffee wilt disease (CWD). Coffee wilt disease, also commonly referred to as tracheomycosis or sometimes

*Corresponding author. E-mail: sammyolal83@gmail.com; kalenzid@gmail.com

carbuncullariosis, is caused by a typical fungal vascular pathogen known as *Fusarium xylarioides* Steyaert. The sexual (perfect) form is *Gibberella xylarioides* (Heim and Saccas, 1950). *F. xylarioides,* which is the conidial (imperfect or anamorph) state of this species was isolated and described by Steyaert from diseased specimens of Excelsa coffee (*Coffea excelsa*) in 1939 (Flood, 1996). Although, Fusarium species have a wide host range, some are quite specific. *F. xylarioides* is a specific coffee pathogen which is rarely found elsewhere (Waller and Holderness, 1997). Tracheomycosis was first reported in *C. excelsa* in the Central African Republic; on robusta coffee (*C. canephora*) in Côte d'Ivoire in 1948, and Congo causing major damage. The disease affects arabica coffee in Ethiopia, while in Uganda it has been attacking only robusta coffee (Adugna et al., t2005). Rutherford (2006) in his inter-simple sequence repeat (ISSR) analysis of *F. xylarioides* isolates from coffee wilt disease infected robusta or arabica coffee observed that these plants were affected by distinct strains. In addition, a study using infection tests in Ethiopia showed host-strain specificity where isolates from Arabica coffee plants were pathogenic only to seedlings of *Coffea arabica* (Adugna et al., 2005). The pathogen survives in the soil. It is difficult to control the pathogen by fungicides. However, the pathogen may be controlled by antagonistic biological control agent such as Trichoderma species which has been reported to control the pathogen by up to 71% *in-vitro* (Alemu, 2012).

Typical symptoms of the disease include curling and yellowing of leaves, defoliation and die-back, blue-black streaks on the wood under the bark, general wilting and death of infected plant (Heim and Saccas, 1950). Unusual symptoms due to other coffee diseases and pests or physiological disorders such as root rot, bark disease; root mealy bug, termites, stem borer; effect of drought and nitrogen deficiency can sometimes be confused with CWD symptoms (http://aces.nmsu.edu/desertblooms/nmsugardening/docs/chap_3/chap3.a.pdf). This can result in misdiagnosis of the disease in the field. It is generally known that symptom expression is a product of the host-pathogen interaction, and host colonization is a prerequisite for disease manifestation (Gaumann, 1950). Existing procedures for recording the incidence of CWD is based only on the expression of the symptoms. In addition there is no indicator that plants can be used to show the presence of pathogen in soil. At present, identification can only be confirmed in the laboratory by plating out infected, surface-sterilized material on appropriate media; while traditional detection from soil is based on baiting techniques since isolation directly from the soil on artificial medium has so far been unsuccessful (Adipala-ekwamu et al., 2001). The agar plate test is lengthy since sporulation is needed for accurate identification. Unfortunately, the identification of fungi based on traditional cultural, morphological and metabolic characteristics may take days and weeks and are laborious, time consuming and require significant technological expertise. This is because different isolates often display characteristics from more than one species (Bowen et al., 1996). For a more complete understanding of the disease, a specific detection technique for *F. xylarioides* in symptomless but infected hosts is essential.

In recent years, molecular and serology techniques of plant disease detection have been well established (Sindhuja et al., 2010). The molecular methods, which are usually PCR based is where a specific base sequence of the genetic material of the pathogen is utilized, while in serological or immunological methods, the microbial protein (antigen) associated with a pathogen is introduced into an animal that produces specific antibodies against the antigen. The techniques are gaining importance due to their specificity, sensitivity and rapidity. Such advanced plant disease detection techniques can provide rapid, accurate, and reliable detection of plant diseases in early stages for economic, production, and agricultural benefits (Sindhuja et al., 2010). In the present paper, we report the identification of the *F. xylarioides* immunodiagnostic target protein that has produced specific polyclonal antibody in rabbit. The specific antibodies can be packaged into a immunodiagnostic procedure and integrated with the current culture based-techniques for better detection of the pathogen both in soil and coffee plant parts. The outcome of the work will be rapidity and simplicity of the test (Sally and Robert, 1988), early detection of CWD pathogen in the host when the infection levels are very low, in-planta proliferation of pathogen, determination of fungal biomass in infected tissues, characterisation of genetic races of the pathogen (Bhuvanendra et al., 2010), based on their protein banding pattern and immuno-reactivity patterns.

The purpose of the study was therefore, to identify a target antigen and validate its corresponding polyclonal antibody for specificity to formulate an immunoassay technique that is simple, fast, highly specific and sensitive for detection of *F. xylarioides* in the soil and plant parts.

MATERIALS AND METHODS

Sample collection

Samples of CWD infected Robusta coffee trees were collected in duplicates from each of the 8 districts in the four agro-ecological farming systems in Uganda where coffee is grown and CWD has been reported. The farming systems were; 1) banana/Coffee System from the districts of Bundibugyo, Mubende, Mukono, and Kalangala; 2) banana/millet/cotton System from Kamuli district; 3) Montane system from Rukungiri and Kabarole districts; 4) pastoral system from Rakai district. The specimens were transported to the laboratory wrapped and labeled individually in paper bags. *F. xylarioides* isolate obtained from a wilted coffee tree at the Coffee Research Institute, Kituza, Mukonoin Uganda was used as a reference.

Fungal isolation and identification

The bark of the stem specimen was carefully removed and small sections (0.5 x 0.5 cm) excised from the intervening regions between discolored wood (lesion tissues) and white healthy wood using a sterile scalpel. The sections (5 to 6) were transferred to plastic Petri dishes and surface sterilized by immersing in 1% v/v sodium hypochlorite solution for 1 to 2 min and rinsed twice in sterile distilled water. The sterilized tissue was then blotted dry by pressing with sterile tissue paper, trimmed to size and placed on tap water agar (TWA) that comprised 20% agar, and incubated at 25°C for three days (Booth, 1971). Emerging fungal hyphae from the wood pieces were sub-cultured on fresh Synthetic Nutrient Agar (SNA) with four pieces of sterile filter paper (1 x 1 cm) placed around the periphery of the set agar to induce sporulation (Nirenberg, 1976) and then on potato sucrose agar (PSA) to encourage development of pigmentation (Booth, 1971). The cultures were incubated under 12 h fluorescence light and dark cycles at 25°C. After 10 days, different *Fusarium* species were identified based on the typical cultural and morphological characteristics of the species as described by Booth (1971). *F. xylarioides* isolate used as gold standard was the one identified at the Coffee Research Institute under the microscope and confirmed at the Global Plant Clinic, CABI Bioscience, UK Centre, England.

Preparation of mycelial homogenates

The different isolates were grown separately in 100 ml Erlenmeyer flask containing 20 ml of potato dextrose broth. The medium was inoculated with four (4 x 4 mm) plugs taken from the periphery of young fungal cultures (5 day old) on PDA/SNA medium. The cultures were incubated stationery in the dark at 20°C for six days. The mycelium was harvested by pouring the culture broth containing fungal hyphae into sterile steel tea strainer. The mycelial mats were finally rinsed thoroughly with de-ionized water blotted dry and then frozen in liquid nitrogen and immediately ground with a mortar and pestle to a fine powder. The powder was suspended in 2 ml of Phosphate Buffered Saline (PBS) containing 0.05% pentylmethyl-sulphonyl fluoride (PMSF) which was added per gram of mycelium ground and centrifuged at 30,000 *g* for 30 min at 4°C, and the supernatant was stored at 20°C until use. The protein content in the supernatant was estimated by the method of Brayford and Flood (1997) using Bovine serum albumin (BSA) as a standard.

Protein analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to assess the purity of the samples, locate the required protein antigen (s) and to prepare proteins for transfer to nitrocellulose membrane for immunobloting (Laemmli, 1970). Soluble protenacious components from mycelial extracts were adjusted to 2 mg/ml in PBS. They were diluted (v:v) in denaturing buffer containing 10% glycerol, 16 of 10% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 12.5% 0.5 M Tris-HCl (pH 6.8) and 5% of 1% v/v bromophenol blue then 47.5% distilled water (Laemmli, 1970). Samples were boiled for 5 min and loaded immediately. Leftovers were stored at -20°C till use. Extracts (10 to 30 µg) were placed in each well. Extracts were stacked in a 4% polyacrylamide gel and resolved according to their molecular sizes on a 10% polyacrylamide gel. Electrophoresis was carried out on a Mini-PROTEIN 11 Electrophoresis Cell (BIO-RAD®, USA). Migration took place at a constant intensity of 120 V and 400 mA at room temperature, until the dye marker had reached the bottom of the gel. The resolved bands were revealed on the gel by staining with 1% Coomassie blue R 250 in fixative (40% methanol, 10% acetic acid) for 2 h at room temperature. The gel was de-stained with three changes of de-staining solution consisting of 40% methanol and 10% acetic acid until the background had been removed and the bands were clearly seen. For better viewing, gel was placed against white light provided by transilluminator (VILBER LOURMAT®, France). Gel was documented by photographing using a digital camera (OLYMPUS D-490 ZOOM®, Germany). The different protein profiles from the other 5 *Fusarium* species were compared and contrasted with that of *F. xylarioides*. Three protein candidates that appeared unique to *F. xylarioides* were of molecular weight 66, 50 and 27 kDa. These were excised from the gel, solubilized in PBS by crushing in chilled mortars, centrifuged and supernatant stored at -20°C until required.

Production of polyclonal antibodies against selected bands

Female New Zealand White rabbits 6 months old were purchased locally and reared in cages. Every selected band extract was adjusted to 100 µg protein in 0.5 ml of PBS to be used per rabbit. An equal volume of Freund's complete adjuvant (FCA) was added and the 1 ml mixture emulsified, and injected by subcutaneous injections into a rabbit at four sites on the back to elicit immune response to produce polyclonal antibodies (Harlow and Lane, 1988). Each rabbit received two or more intramuscular booster immunizations of 50 µg protein/ 0.5 ml PBS emulsified in 0.5 ml Freund's incomplete adjuvant (FIA) after every fortnight. This continued until a required titre was attained. Bleeding was 10 days after boosting. Blood was obtained from the marginal ear vein following the shaving around its posterior edge and cleaning with a cotton wool soaked in 70% ethanol. The blood collected was allowed to clot at room temperature for 1 h then stored in the fridge (4°C) overnight then centrifuged at 2,500 *g* for 30 min to separate the serum. The separated sera were stored in 200 µL aliquots in 0.5 ml tubes at -20°C until required for antibody analysis.

Dot blot analysis

This procedure was performed as a quick check for the positivity and specificity of the antisera from rabbits. Samples of all the soluble protein extracts from seven *Fusarium* species were prepared in PBS pH 7.4 to a final concentration of 2 mg protein per ml. Ten microlitres of this solution was spotted onto strip of nitrocellulose membrane (0.45 mm pore size; Bio-Rad Laboratories, Richmond, California) and air dried for 5 min. Non-specific binding sites were blocked with 5% skimmed milk in PBS by incubating for 2 h at room temperature while rocking. After washing three times, by shaking for 5 min during each wash in PBS-Tween 20 (PBS containing 0.05% tween 20), the membrane was incubated for 1 h in the test rabbit immune sera at a dilution of 1:100 as primary antibodies and again washed as described above. The washed membrane was then probed with peroxidase-conjugated goat-anti rabbit IgG as secondary antibodies for 45 min and washed 3 times with PBS-Tween 20 as above. The positive reaction were visualized by use of diaminobenzitine (DAB) solution (1.3 mM DAB in 0.01M Tris-HCl containing 0.004% H_2O_2) as the substrate. After the appearance of the colour, the membranes were washed with distilled water for several times to stop the reaction. The development of well-defined brown dots on the nitrocellulose membrane was considered as positive.

Immunoblotting (Western blot) analysis

This was done to confirm the specificity of the antibodies: 20 uL of crude protein extracts from the eight *Fusarium* species were first resolved over 10% SDS-PAGE as described above in duplicate, one for Commasie blue staining for contrasting with the Western

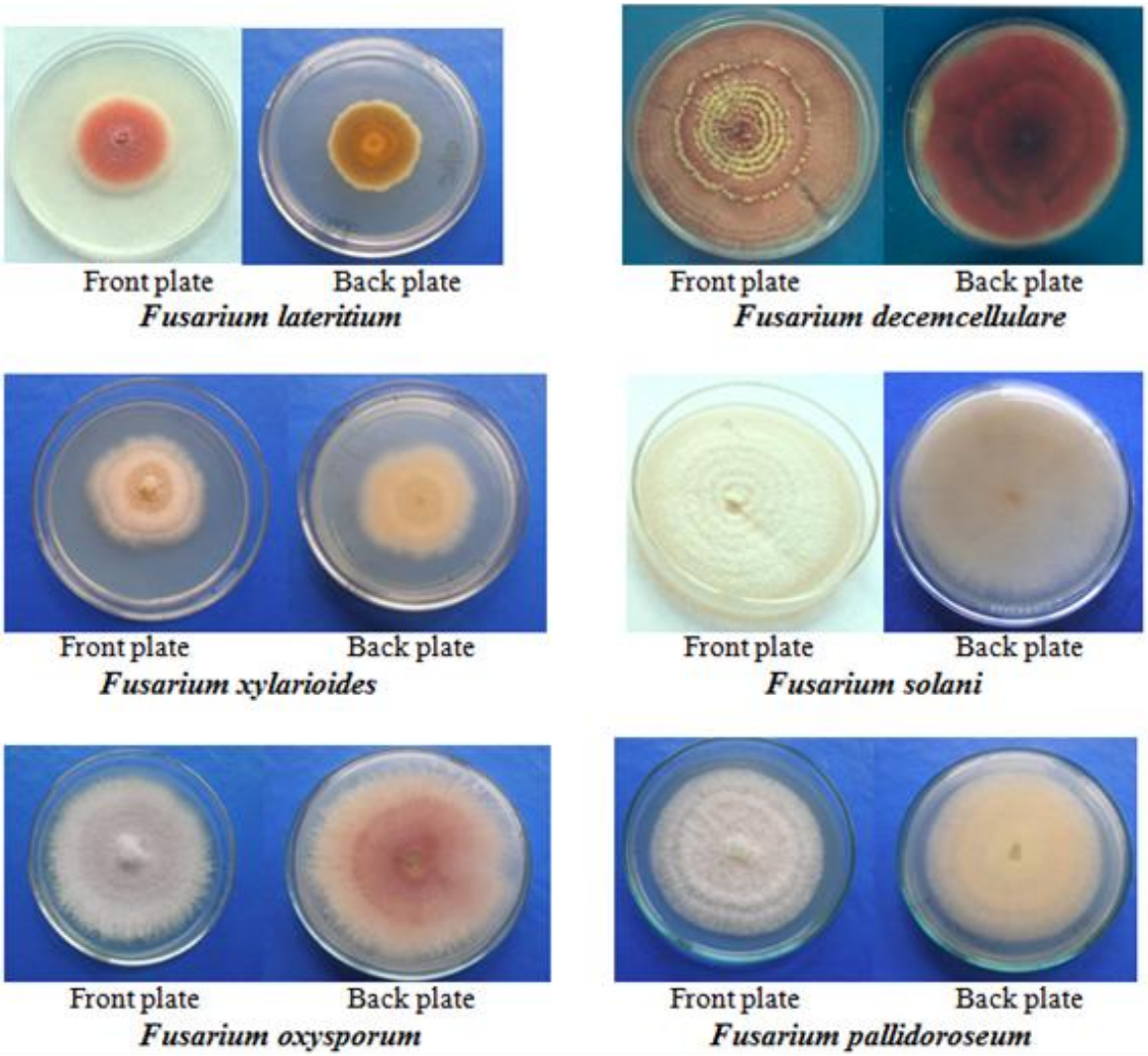

Figure 1. Culture pigmentation of different *Fusarium* species. Cultures grown on Potato Dextrose Agar (PDA) and induced pigmentations peculiar to each *Fusarium* species and used as an aid to correct identification. (Booth,1971). Culturing was done at room temperature at 12 h light / 12 h dark cycles for 10 days.

blot result while the other one was for protein transfer to nitrocellulose membrane. Resolved proteins were transferred onto nitrocellulose paper for 1 h at 100 V and 250 mA current flow using transfer buffer that comprised 25 mM Tris (3.03 g), 192 mM glycine (14.4 g), 20% v/v methanol (200 ml and distilled water 800 ml) and a pH 8.5. Bio-ice unit (Bio-Rad, USA) was included to provide cooling during the transfer that was carried out with continuous stirring using a magnetic stirrer. After the transfer, non-specific binding sites on the membrane were blocked with blocking buffer (5% skimmed milk powder in PBS) for 2 h at room temperature with constant rocking or kept overnight at 4°C. Washing followed 3 times with PBS containing 0.05% Tween 20. Primary antibody (immune serum) was added at a dilution of 1 part immune serum to 100 parts PBS and incubated 45 min at room temperature as above to probe the blot. Washing was as described above. Secondary antibody (Horseradish peroxidase conjugated goat anti-rabbit IgG at a dilution of 1:8000 in PBS and incubated for 1 h at room temperature as above. Washing followed. Colour development to reveal positivity and specificity of the immune serum (antibody) was done by adding 10 ml of 0.01 M Tris-HCl containing 6 mg of diaminobenzitine (DAB) and 10 uL of 30% pre-mixed sodium peroxide (H_2O_2). Brown colour development took place within a few seconds and reaction was stopped by washing the membrane with distilled water. The development of well-defined brown bands on the nitrocellulose membrane was considered as positive.

RESULTS

Culture pigmentation of different *Fusarium* species

The isolates used in the study were successfully obtained mainly from diseased coffee plants. Each *Fusarium* species exhibited peculiar culture pigmentation (Figure 1) that was made; use of correct identification alongside spore morphologies (Booth, 1971). Different *Fusarium*

Figure 2. Antibody specificity testing by Dot blot analysis. A, *Fusarium oxysporum* extract spot; B, *Fusarium solani* extract spot; C, *Fusarium xylarioides* extract spot; D, *Fusarium lateritium* extract spot; E, *Fusarium decemcellulare* extract spot; F, *Fusarium moniliforme* extract spot; G, *Fusarium pallidoroseum* (*Semitectum*) antigen spot. The blot was developed with antiserum diluted 100 folds. The positive reaction were visualized by use of diaminobenzitine (DAB) solution (1.3 mM DAB in 0.01 M Tris-HCl containing 0.004% H_2O_2) as the substrate. The development of well-defined brown dots on the nitrocellulose membrane indicated positive reaction.

species produced mycelial mat with pigments peculiar to a species and of varying densities. *F. xylarioides, Fusarium solani* and *Fusarium oxysporum* produced soft mycelial mat and hence eased protein extraction. *Fusarium decemcellulare, Fusarium semitectum* and *Fusarium lateritium*, however, produced tougher mycelium that was more difficult to homogenize using both chilled mortar in liquid nitrogen and ultra-sonicator and produced less amount of protein compared to the soft mycelia.

Reactivity and specificity of the immune sera

Antisera that rose to soluble mycelial proteins or candidate proteins in selected bands of *F. xylarioides* were tested for cross reactivity with antigens from five other *Fusarium* species. Antisera of 50 and 66 kDa band proteins were dropped due to inconsistence in reactivity with different protein extraction batches and antibody titre being low resulting in weak bands and non-specific reactions. In contrast, antisera to proteins in the 27 kDa band reacted positively only with *F. xylarioides* isolates. Pre-immunization serum was included as a negative control. As expected, the pre-immune serum did not recognize any of the test antigens.

Dot blot analysis

This was done to quickly assess the production and specificity of antibodies. The antibodies rose against 27 kDa protein which recognized only the *F. xylarioides* spot and not any of the other *Fusarium* species as shown in Figure 2.

Immunoblotting (Western blot)

Western blot was performed to confirm the specificity of the immune sera. The results confirmed that polyclonal antibody raised against the 27 kDa antigen indeed recognizes only the target CWD pathogen, *F. xylarioides* (Figure 3B). Western blot analysis, therefore, revealed no cross-reactivity with non-target antigens of other *Fusarium* species.

Diagnostic potential of the 27 kDa antigen

Antigenic variations among *F. xylarioides* isolates was evaluated using anti -27 kDa protein anti-serum against eight *F. xylarioides* isolates taken from four farming agro-ecological systems in Uganda where CWD exists. The antiserum recognized all the isolates tested with prominent bands proving its potential to detect the pathogen from any part of the country (Figure 4).

DISCUSSION

The aim of the study was to identify and validate a diagnostic immunogen (antigen) from *F. xylarioides* mycelial extracts that can be used to generate specific polyclonal antibodies for the detection of the pathogen in test materials. Accurate and specific antigen to the pathogen has been identified. The potential of such immunodiagnostic tools to detect colonization of roots by several soil-borne fungal pathogens has been demonstrated (Priestley and Dever, 1993; Srivastava and Arora, 1997). This is the first report describing the use of a serological technique for the detection of *F. xylarioides.* Six *Fusarium* species common to coffee as saprophytes or true pathogen (Hakiza and Webesa, 1997; Serani, 2000) namely *F. xylarioides, F. solani, F. oxysporum, F. decemcellulare, Fusarium Palldoroseum*, and *F. Lateritium* identified according to Nirenberg (1976) and Booth, (1971) were used to identify the target antigen in this study. Soluble proteins, crude cell component, fungal homogenate, culture fluids, and ribosomal proteins from fungal pathogens have been used to raise antibodies (Jamaux and Spire, 1994; Priestley and Dever, 1993;

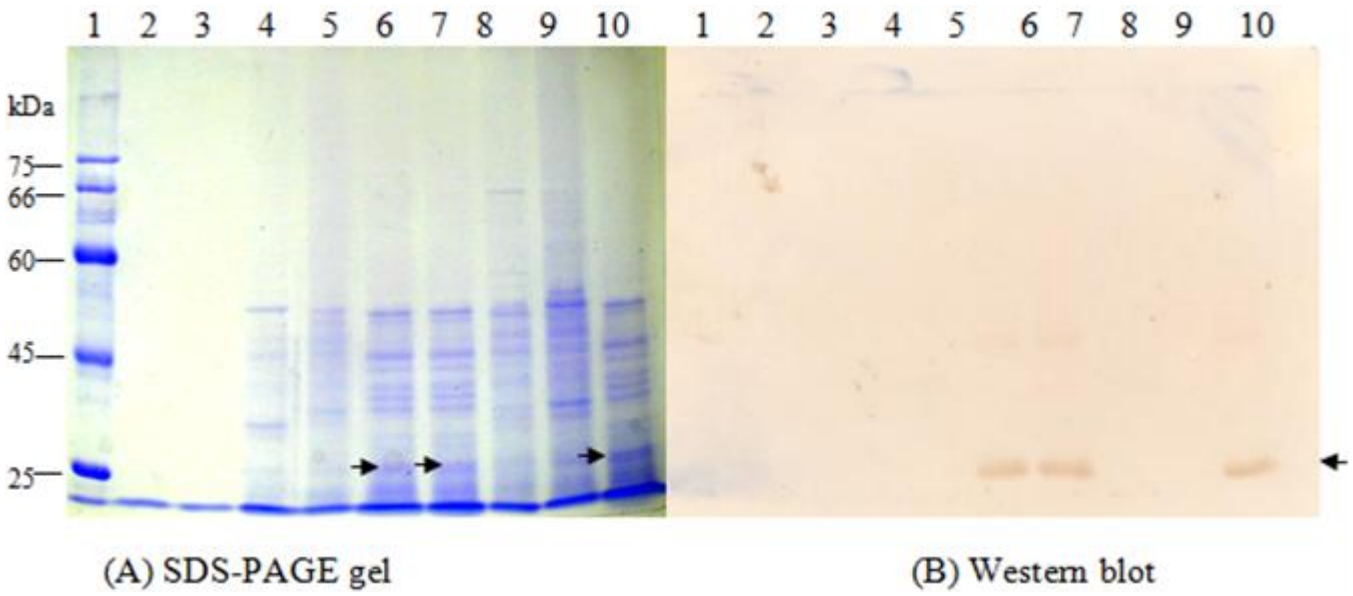

Figure 3. Antibody specificity confirmation by immunoblotting. Lane 1, molecular mass standard; lane 2 and 3, *Fusarium oxysporum*; lane 4, *Fusarium lateritium*; lane 5, *Fusarium pallidoroseum* (*semtectum*); lane 6, *Fusarium xylarioides*; lane 7, *Fusarium xylarioides*; lane 8, *Fusarium decencellulare*; lane 9, *Fusarium solani*; lane 10 *Fusarium xylarioides*. (B) SDS-Polyacrylamide electrophoresis and blotted. The blot was developed with antiserum diluted 100 - fold and conjugate 8000-fold. Column on the left listed the molecular masses obtained for the various bands. Arrowed is the 27 kDa protein (A) with its corresponding Western Blot (B).

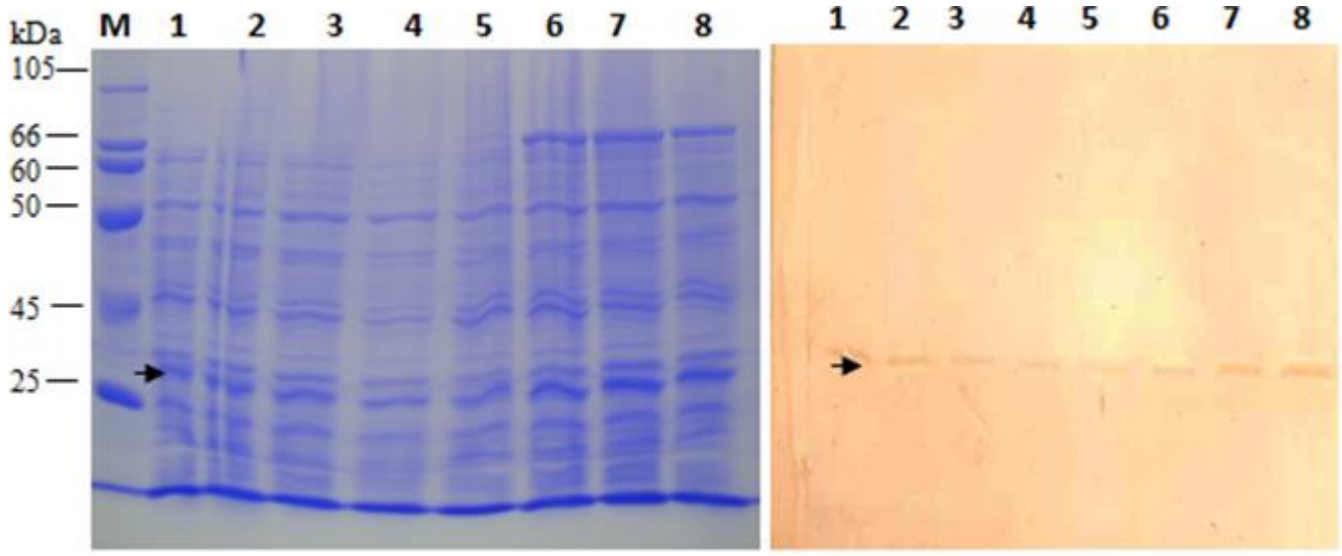

Figure 4. Recognition of a wide range of isolates by the anti- *F. xylarioides* 27 kDa band antiserum. Origin of different *Fusarium xylarioides* isolates. Lane M, Molecular marker; lane 1, Bundibugyo; lane 2, Mubende; lane 3, Mukono; lane 4, Kalangala; lane 5, Kamuli; lane 6, Rukungiri; lane 7, Kabarole; lane 8, Rakai. Samples were separated by SDS-polyacrylamide electrophoresis and blotted. The blot was developed with antiserum diluted 100-fold. Arrows indicate bands of apparent molecular mass 27 kDa (A) and corresponding blot (B).

Srivastava and Arora, 1997). In this study, part of the soluble mycelial extract was used as the antigen for antibody production. The result of analysis of the total mycelial extract from the six *Fusarium* species by SDS-PAGE enabled the identification of protein bands peculiar to *F. xylarioides*. The protein bands that appeared peculiar to the pathogen were those of molecular masses 27, 50 and 66 kDa. Protein bands of 50 and 66 kDa were

dropped from the study due to their inconsistency in reactivity with different protein extraction batches.

In addition, they induced low antibody titre and weak bands on western blots. The study therefore identified mycelial extract fraction of 27 kDa as the best antigen to induce antibodies for specific detection of *F. xylarioides*. The result is in agreement with a finding that electrophoresis of proteins can be a useful tool for identification and characterization of the genetic differences among *Fusarium* species (Bhuvanendra et al., 2010, Heidi et al., 2011). The work by Heidi et al. (2011), however, identified protein of 45.2 kDa as specific to *F. xylarioides* isolated from sugar beet and wheat. This variation in protein markers for the same species could be explained by observed variation in the pathogen races (Rutherford, 2006) host - race specificity (Adugna et al., 2005) or geographical area impact on the genome regardless of the host (Vitale et al., 2011). Since protein polymorphism has been noted to reflects the genetic background of the microorganisms (Shaw, 1965), those specific proteins could be linked to host - race specificity.

No attempt was made to further purify the protein by cross - absorption technique since some workers have reported that the procedure rarely improves the specificity of the antiserum raised to such protein (Srivastava and Arora, 1997) or even it specificity is improved, sensitivity is reduced (Jamaux and Spire, 1994). The specificity of the antiserum to the 27 kDa protein band was evaluated by dot blot and western blot analyses. The antiserum reacted strongly only with *F. xylarioides* and no cross-reaction with any test antigens was observed (Figure 3). The antibodies to this antigen, therefore, provide an ideal probe for the detection of the fungus that causes CWD. It is unlikely that the 27 kDa antigen was a simple protein but a glycoprotein since previous studies has demonstrated that fungal antigens that were specific have always been found to be glycoprotein (Deway et al., 1990). The work also indicated that immunization of rabbits with high-molecular-weight proteins induce antiserum that is non-specific and recognize all test antigens. A possible explanation for this is that the high-molecular-weight protein/glycoprotein molecules are immune-dominant and block the development of antibodies to specific molecules as suggested by Priestley and Dever (1993). Immunization of rabbits with a low-molecular-weight (<30 kDa) reduced considerably the number of cross-reacting antigens. The result indicates that the cross-reactive antigens lie mainly in the high-molecular-weight protein/glycoprotein fraction of mycelial extracts. The present study has produced a specific anti-27 kDa protein antiserum and provides a significant breakthrough in the development of immune-assay procedure for sensitive, specific and rapid diagno-sis of CWD. Species specificity of the anti-27 kDa protein antiserum, therefore, disagrees with earlier work done by some workers which indicated that polyclonal antibodies cannot differentiate between species and more so between *Fusarium* species (Srivastava and Arora, 1997); and that polyclonal antisera raised to crude mycelial extract of one species, have often been found to be generally genus-specific (Jamaux and Spire, 1994). Cloning and expressing the 27-kDa protein can undoubtedly improve the procedure of antigen preparation, thereby making it more cost-effective. Further studies will be concerned with the evaluation of its practical application for epidemiological studies.

The use of immunoassay for identification is based on specific epitopes carried on specific protein/glycoprotein. Fungal proteins/glycoprotein elicits an immune response when introduced into a higher animal, typically a rabbit, resulting in antibody production. Dot blot and Western blot analyses were used to evaluate the specificity of the antibodies produced in this study. The dot-immunobinding assay, a modification of the ELISA using a nitrocellulose membrane as a test matrix, was rapid, specific, sensitive and easy to perform for quick screening for no sophisticated electrical equipment is required as a positive reaction and which is observed by eye with reliability. With Western blotting analysis, some workers have used it to detect either antibodies or antigens and has been used successfully for immunodiagnostic of a variety of parasitic infections (Eamsobhana et al., 2004). The test has been noted as being more specific than the traditional colorimetric ELISA using crude antigens because the band pattern on the immunoblot can be interpreted as positive (reactive) or negative (non-reactive) (Eamsobhana et al., 2004) hence the specificity of the immune sera produced was evaluated with reliability.

For the 27 kDa protein to be considered as species-specific, its presence in all *F. xylarioides* isolates across the country was evaluated. Western blot analysis against 8 *F. xylarioides* isolates from four agro-ecological zones in Uganda where CWD exists using anti- *F. xylarioides* 27 kDa protein confirmed that the protein antigen is indeed species specific (Figure 4). There is therefore no antigenic variation with respect to the 27 kDa antigen in the pathogen. The result is in agreement with DNA characterization work by Janzac et al. (2004); Rutherford (2006) showing no interspecies variations in the pathogen causing CWD on Robusta coffee in Uganda, Tanzania and the DRC. This suggests that the antiserum can be used universally to detect CWD pathogen on Robusta coffee.

Conclusion

In conclusion, the species specificity of the anti- *F. xylarioides* 27 kDa protein is very encouraging for the production of a monospecific antiserum. On the basis of the result obtained in the present study, we can conclude that the 27 kDa *F. xylarioides* protein is species specific to CWD pathogen and is present in all isolates so far

studied in Uganda; and secondly, the higher the molecular mass of the protein, the less specific the antisera produced becomes. Development of monoclonal antibodies against the 27 kDa protein would enhance specificity, homogeneity and production of the antibodies. There is also need to develop double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for quick screening of large samples.

Conflict of Interest

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

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Discrimination of oats (*Avena sativa* L.) cultivars using isozyme markers

S. Sumathi* and P. Balamurugan

Department of Seed Science and Technology, Tamil Nadu Agricultural University, Coimbatore-641 003, India.

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The present investigation was carried out to discriminate the oat cultivars based on isozymic banding patterns. The pattern of isozymes such as peroxidase, polyphenol oxidase, esterase and amylase in the selected 11 oats cultivars were studied. The enzyme extracts prepared from seven days aged seedlings of each cultivars were analyzed through polyacrylamide gel electrophoresis (PAGE) and the cultivars were differentiated based on the presence or absence of bands and intensity of bands in each isozymes. In peroxidase, totally four bands were recorded, and among these, three bands were common for all cultivars; the band at Rf value 0.944 showed polymorphism. In polyphenol oxidase, totally three bands were recorded; the absence of band at Rf value 0.797 in OS 7, HJ 8 and OL 9 are used for differentiation. In esterase, totally six bands were noticed, the presence of band at Rf value 0.857 in OS 7 and HJ 8 are used for discrimination and in amylase, totally seven bands were recorded. Among the cultivars, variations in band intensity helped for cultivar identification.

Key words: Oats, polyacrylamide gel electrophoresis (PAGE), isozymes, cultivar identification

INTRODUCTION

Oats is an important cereal cum forage crop. It has rich medicinal and nutritional value and is being used as food by the diabetic patients, since the grains are filled with cholesterol fighting soluble fibre (Singh et al., 2003). The increased economic importance of the crop has stimulated the development of new cultivars. At present, large numbers of cultivars are available for commercial cultivation. Therefore, identification of cultivars became very important in seed certification and crop breeding programmes. In traditional method of cultivar identify-cation, morphological features are commonly used for identification. For morphological characterization, the plant must be grown to flowering or fruiting stage, which is a laborious and tedious process (Gottschalk, 1985). Moreover, plant and seed morphology has been unreliable since morphological characters can be greatly influenced by environment conditions (Geeta and Kanwar, 2006). With increase in the number of varieties of each crop, it is very difficult to distinguish the varieties on the basis of morphological characters alone. This has led to the development of the new stable parameters such as use of their genetic material as a tool for cultivar identification. Electrophoresis is a process of separation of different biomolecules (protein and isozymes) under the influence of electric field and has been successfully applied for the identification of cultivars (Vishwanath et

*Corresponding author. E-mail: anbumathi_003@yahoo.co.in.

al., 2011).

The most widely used biochemical markers in plant breeding and applied genetics are isozymes. It is polymorphic and more stable than morphological traits across environments, therefore these accurate and inexpensive biochemical markers has been used extensively to characterize the cultivars (DiRenzo et al., 2001). The isozyme banding patterns are not so complex, when compared to bands of proteins. The adequate allozymeric variability of cultivars helps in differentiation of very closely related species. Hence, the studies were formulated to discriminate 11 oats cultivars using polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Seed materials

The genetically pure 11 oats cultivars *viz.,* Sabzar, Kent, HFO 114, OS 6, UPO 212, OS 7, HJ 8, UPO 94, OL 9, JHO 822 and OL 88 obtained from Pant University of Agriculture and Technology, Uttar Pradesh were used for characterization based on profile of isozymes such as peroxidase, polyphenol oxidase, esterase and amylase.

Preparation of sample

Five hundred milligram of plant tissues, which includes root and shoot was taken from seven days old seedlings and macerated with pestle and mortar using 750 µl of ice cold 0.1 M phosphate buffer (pH 7) for peroxidase, 0.01 M phosphate buffer (pH 7) containing 1% non-ionic detergent (Tween 80) for polyphenol oxidase, 10 mM sodium phosphate buffer (pH 6.9) for esterase and 50 mM Tris HCl buffer (pH 7) for amylase. The slurry obtained was centrifuged at 4°C at 12,000 rpm for 20 min. The supernatant was transferred into a fresh eppendorf tube. This formed the crude extract for enzymes analysis.

Electrophoresis

The extracted isozymes were separated using PAGE in a discontinuous buffer system at a monomer concentration of 30% acrylamide stock gel solution using 7.5% separating gel containing 1.5 M Tris HCl (pH 8.8) and 4% stacking gel containing 0.5 M Tris HCl (pH 6.8) as prescribed by Dadlani and Varier (1993) for peroxidase and polyphenol oxidase, Smith et al. (1970) for esterase and Lin et al. (1988) for amylase with slight modifications. Sample of 30 and 20 µl of sample buffer (5X concentration) containing bromophenol blue (tracking dye) was loaded in each well. Electrode buffer was filled in buffer tank and the space between the two glass plates. The electrophoresis unit was kept in a refrigerator to dissipate the heat produced during electrophoresis. The voltage was set at 125 V and ran till the tracking dye reach the end of the gel. Then the gel was transferred into the corresponding staining solutions.

Staining and fixing

Peroxidase

One hundred milligram of benzidine dissolved in 1 ml of acetic acid and made to 100 ml with acetate buffer (0.1 M) was added to the gel and incubated for 30 min at 35°C in dark with constant shaking. After 30 min, 1 to 2 ml of hydrogen peroxide was added drop by drop till the blue colour bands appeared and then the reaction was stopped by transferring the gel to 7% acetic acid solution.

Polyphenol oxidase

One hundred milligram of p-phenylene diamine (0.1%) and 0.1 g of Catechol (10 mM) were mixed in 100 ml of 0.1 M potassium phosphate buffer (pH 7.0) and the gel was equilibrated for 30 min in the solution. After the appearance of brown coloured bands, the reaction was stopped by transferring the gel into 7% acetic acid solution.

Esterase

The gel was incubated in the solution containing 2.8 g of sodium dihydrogen phosphate, 1.1 g of disodium hydrogen phosphate, 0.2 g of Fast blue RR salt and 0.03 g of Alpha-naphthyl acetate dissolved in 200 ml of distilled water for 30 min preferably in the dark at 37°C. After appearance of brown coloured bands, reaction was ceased by immersing the gel in a solution mixture of methanol: water: acetic acid: ethyl alcohol in the ratio 10:10:2:1.

Amylase

The gel was incubated in 1% starch solution buffered to pH 5.0 using 0.1 M acetate buffer at 37°C for 30 to 40 min. After incubation, the gel was soaked in potassium iodide - iodine solution (43.3 mM potassium iodide and 5.7 mM Iodine). After the appearance of translucent bands in dark blue background, the gel was photographed.

Evaluation and documentation

The distance moved by the tracking dye from the point of loading was measured on the gel. Then the distance traveled by each band was also measured. The complete gel imprints were made on the transparency sheets to determine their intensity. Relative front (Rf) of each band was calculated as follows:

$$Rf = \frac{\text{Distance travelled by the band}}{\text{Distance travelled by the tracking dye}}$$

Bands were numbered in the order of increasing Rf values. Apart from this, recording presence or absence of a band and the intensity of the band in each cultivar was critically observed for discriminating the cultivars.

RESULTS AND DISCUSSION

Isozyme markers are invaluable tool for cultivar identification, their expression is almost exclusively of the genetic makeup of the plant, and therefore rarely affected by environmental conditions; their patterns are highly reproducible. Therefore, it is feasible to use variation in isozymes banding pattern for varietal characterization (Bretting and Widrlechner, 1995). In the present study, the enzymes were extracted from eleven oats cultivars to

Table 1. Intensity and relative front of peroxidase in seedlings of oats cultivars.

Band Number	Rf value	Cultivars										
		Sabzar	Kent	HFO 114	OS 6	UPO 212	OS 7	HJ 8	UPO 94	OL 9	JHO 822	OL 88
1	0.766	++	++	++	++	++	+++	++	++	++	++	+
2	0.802	++	++	++	++	++	+++	++	+++	++	++	+
3	0.851	+++	+++	+++	++	++	+++	++	+++	+++	+++	++
4	0.944	+++	+++	+++	++	++	-	-	+++	-	-	++

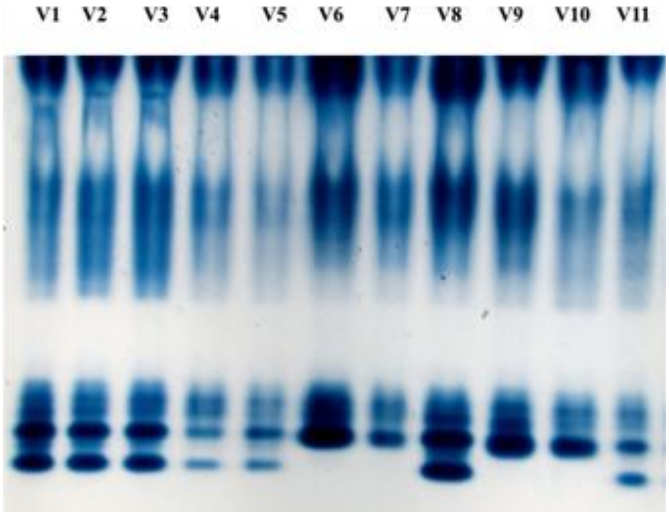

Figure 1. Peroxidase isozyme profile of oats cultivars. V1, Sabzar; V2, Kent; V3, HFO 114; V4, OS 6; V5, UPO 212 ; V6, OS 7; V7, HJ 8; V8, UPO 94; V9, OL 9; V10, JHO 822; V11, OL 88.

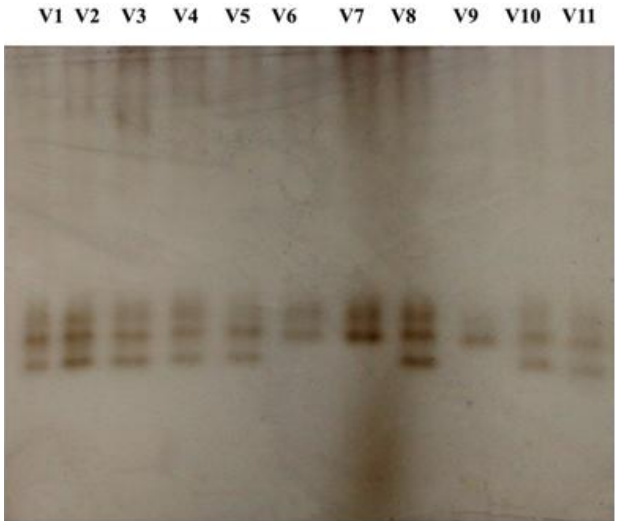

Figure 2. Polyphenol oxidase isozyme profile of oats cultivars. V1, Sabzar; V2, Kent; V3, HFO 114; V4, OS 6; V5, UPO 212 ; V6, OS 7; V7, HJ 8; V8, UPO 94; V9, OL 9; V10, JHO 822; V11, OL 88.

analyze the peroxidase, polyphenol oxidase, esterase and amylase through PAGE method. The electrophoretic analyses of peroxidase revealed that totally, four bands were observed, at Rf value ranging from 0.766 to 0.944. The presence or absence of bands and their intensity variation was taken as the main criteria for cultivar identification. All four bands were observed in cv. Sabzar, Kent, HFO 114, OS 6, UPO 212, UPO94 and OL 88 with different intensity. The remaining cultivars recorded three numbers of bands. The absence of band at Rf value 0.944 in OS 7, HJ 8, OL 9 and JHO 822 was used for discrimination of these cultivars from others (Plate 1 and Table 1). Many workers have reported the successful use of seedling peroxidase as in the case of the present study. Santhy et al. (1998) in rice, Nagaraja et al. (2000) in sorghum, Roy et al. (2001) in grass peas, Manonmani et al. (2004) in Indica rice and Praveena (2005) in muskmelon reported the usefulness of seedling peroxidase in varietal discrimination.

The electrophoretic analysis of polyphenol oxidase isozyme expressed totally three numbers of bands. The detailed electrophoregram and results of these is presented in Figure 2 and Table 2. The expression of bands at Rf value range from 0.681 to 0.797. All the three bands were observed in cultivars Sabzar, Kent, HFO114, OS6, UPO 212, UPO94, JHO822 and OL 88. Cultivars OS 7 and HJ 8 expressed two numbers of bands. Only one band was recorded in OL9 and remaining two bands were absent, this helps for identification. Thangavel (2003) in sorghum and Eevera (2003) in rice differentiated the cultivars based on polyphenol oxidase banding pattern. The banding pattern and Rf values of esterase isozyme is presented in Figure 3 and Table 3. Totally, six bands at Rf value ranging from 0.426 to 0.857 were expressed. The maximum of five bands were observed in UPO 94. The minimum two bands were observed in HJ8 and OL 88. Unlike peroxidase and polyphenol oxidase, there was no common band in esterace. Varier et al. (1995), Prasada Rao et al. (2001) in pearl millet, Choer et al. (1999) in beans, Dvoracek et al. (2001) in spelt wheat, Arunkumar et al. (2007) in pearl millet and Rakshit et al. (2011) in maize reported that esterase is considered to be one of the most suitable enzyme systems for differentiating the cultivars. The

Table 2. Intensity and relative front of polyphenol oxidase in seedlings of oats cultivars.

Band number	Rf value	Cultivar										
		Sabzar	Kent	HFO 114	OS 6	UPO 212	OS 7	HJ 8	UPO 94	OL 9	JHO 822	OL 88
1	0.681	++	++	+	+	+	+	+++	+++	-	+	+
2	0.730	++	++	++	++	++	++	+++	+++	++	++	+
3	0.797	++	++	++	++	++	-	-	+++	-	++	+

Table 3. Intensity and relative front of esterase in seedlings of oats cultivars.

Band number	Rf value	Cultivars										
		Sabzar	Kent	HFO 114	OS 6	UPO 212	OS 7	HJ 8	UPO 94	OL 9	JHO 822	OL 88
1	0.426	++	+++	+	++	+++	+++	++	+++	++	+++	-
2	0.662	+	+	+	-	+	-	-	+	-	-	-
3	0.769	-	-	-	+	-	-	-	+	-	-	-
4	0.772	+	+	+	+	+	-	-	+	+	+	+
5	0.836	+	+	+	-	+	+	-	+	+	+	+
6	0.857	-	-	-	-	-	+	+	-	-	-	-

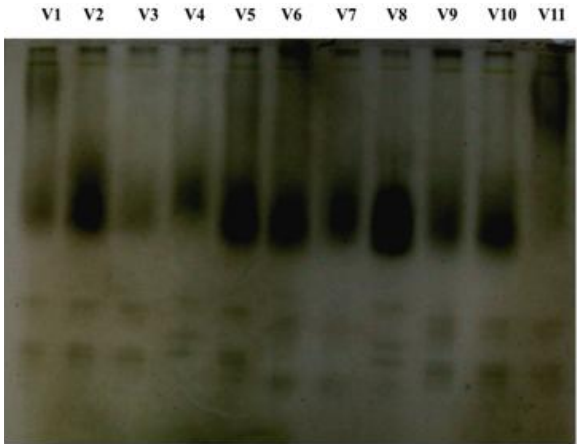

Figure 3. Esterase isozyme profile of oats cultivars. V1, Sabzar; V2, Kent; V3, HFO 114; V4, OS 6; V5, UPO 212 ; V6, OS 7; V7, HJ 8; V8, UPO 94; V9, OL 9; V10, JHO 822; V11, OL 88.

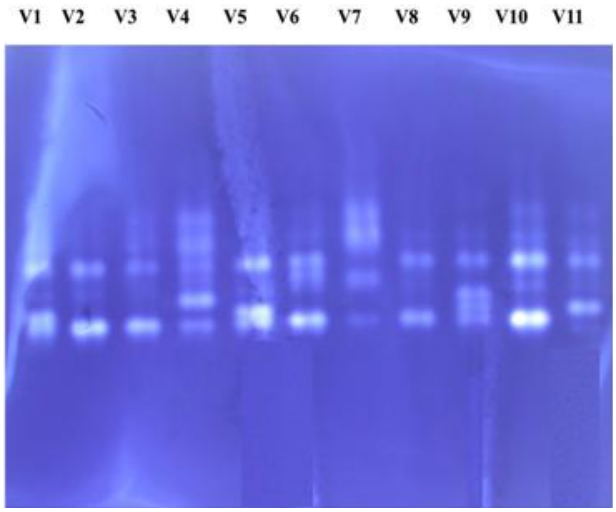

Figure 4. Amylase isozyme profile of oats cultivars. V1, Sabzar; V2, Kent; V3, HFO 114; V4, OS 6; V5, UPO 212 ; V6, OS 7; V7, HJ 8; V8, UPO 94; V9, OL 9; V10, JHO 822; V11,OL 88.

banding pattern of amylase isozyme has been reported in Figure 4. The Rf value were expressed in Table 4.

Totally, seven bands of low, medium and high intensity bands (Rf value ranging from 0.406 to 0.599) were observed. The maximum of six bands appeared in OS6 and OL 9 and minimum of three bands were expressed in Kent; the variations in banding intensity are used for cultivar differentiation. The similar types of identification of cultivars were reported by Diwan and Shenoy (2001) in rice and Zhang et al. (2004) in barley.

From the present study, it may be inferred that in all the isozymes the specific banding pattern helps for varietal differentiation. Among the four isozymes, peroxidase expressed the clear banding pattern when compared to other isozymes. The differentiation of cultivars based on isozymes electrophoresis was easy and simple method when compared to protein, because most allozymes represent codominant loci distribution according to Mendelian laws of inheritance and many loci are being expressed at all stages of the life cycle (Hamrick, 1989). Isozymes detect genetic changes in coding regions of the genome, which results in changed amino acid sequences. These changes in amino acid composition can easily be resolved using polyacrylamide gel electrophoresis

Table 4. Intensity and relative front of amylase in seedlings of oats cultivars.

Band number	Rf value	Cultivars										
		Sabzar	Kent	HFO 114	OS 6	UPO 212	OS 7	HJ 8	UPO 94	OL 9	JHO 822	OL 88
1	0.406	-	-	+	++	-	-	++	-	+	++	+
2	0.445	-	-	+	++	-	+	++	+	+	++	+
3	0.469	+++	++	++	++	+++	++	-	++	++	+++	++
4	0.495	+	+	-	+	-	++	++	+	-	+	-
5	0.553	-	-	-	+++	+	-	-	-	++	-	-
6	0.577	++	-	-	-	+++	-	-	-	++	-	++
7	0.599	++	+++	+++	++	+++	+++	+	++	++	+++	+

+ = Low intensity; ++ = medium intensity; +++ = high intensity.

(Melchinger, 1990). The advantage of use of isozymes as genetic markers are the alleles of most isozyme markers interact in co dominant fashion and facilitate the identification of heterozygotes from homozygotes and nonepisatic expressions of isozymes permit to analyze an infinite number of isozyme markers simultaneously (Tanksley and Rick, 1990).

Conflict of Interests

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

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Full Length Research Paper

The genetic diversity and population structure of common bean (*Phaseolus vulgaris* L) germplasm in Uganda

Dennis Okii[1,2]*, Phinehas Tukamuhabwa[1], James Kami[3], Annet Namayanja[2], Pamela Paparu[2], Michael Ugen[2] and Paul Gepts[3]

[1]Makerere University, College of Agricultural and Environmental Sciences, Department of Crop production. P.O Box 7062, Kampala, Uganda.
[2]National Crops Resources Research Institute-Namulonge, P.O. Box 7084, Kampala, Uganda.
[3]University of California, Department of Plant Sciences/MS1, Section of Crop and Ecosystem Sciences, 1 Shields Avenue, Davis, CA 95616-8780, USA.

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The knowledge and understanding of the genetic variability of common bean (*Phaseolus vulgaris* L.) germplasm is important for the implementation of measures addressed to their utilizations and conservation. The objective of this study was to characterize common bean in Uganda using polymorphic molecular markers for use in hybridization and variety development. Genomic DNA was extracted from plants at the first trifoliate leaf stage growing in pots using the modified cetyl-trimethylammonium bromide (CTAB) method. The gene pool membership (Andean vs. Mesoamerican) for each accession was established with the phaseolin marker. Simple sequence repeat (SSR) alleles were separated by capillary electrophoresis that provided further information on the organization of genetic diversity. The Andean and Mesoamerican genotypes were present in similar frequencies (51 vs. 49%, respectively). All SSR markers tested were polymorphic with mean polymorphism information content (PIC) of 0.8. The model-based cluster analysis of SSR diversity in the STRUCTURE software found three sub populations (K3.1, K3.2 and K3.3) genetically differentiated with moderate Wrights fixation indices (F_{ST}) values 0.14, 0.12 and 0.09, respectively and many cases of admixture. The STRUCTURE result was confirmed by principal coordinate analysis (PCoA) which also clustered beans in three groups. Most Andean genotypes were included in K3.1 and Mesoamerican genotypes belonged to the K3.2 and K3.3 subgroups. This study sets the stage for further analyses for agronomic traits such as yield, resistance to biotic and abiotic stresses and the need for germplasm conservation.

Key words: Phaseolin, Simple sequence repeat (SSR), hybridization, wrights fixation index (F_{ST}), structure.

INTRODUCTION

The domesticated *Phaseolus vulgaris L.* (2n = 2x = 22) consists of two major gene pools, one originating among wild beans ranging from northern Mexico to Colombia (Mesoamerican gene pool) and the other descending from wild beans distributed from southern Peru to north-western Argentina (Andean gene pool) Freyre et al., 1996). The common bean has a widespread distribution on many continents such as Mesoamerica, South

America, Europe and Africa. It reached Uganda presumably in the 18th century via the East African coast (Gepts and Bliss, 1988). Currently, the gene pool of the domesticated species is organized into four Mesoamerican and three Andean eco-geographical races based on morphological, agronomic and ecological grounds (Singh et al., 1991a; Beebe et al., 2001). In the Andean gene pool, the races are Nueva Granada, Peru, and Chile, while in the Mesoamerican gene pool, they are Durango, Guatemala, Jalisco, and Mesoamerica (Blair et al., 2006; Diaz and Blair, 2006). The distinction the Andean and Mesoamerican gene pools is achievable, with the *Phaseolin* - marker (Kami and Gepts, 1994; Burle, 2008).

Microsatellite markers (SSRs) have also been used in common bean to construct genetic reference maps (Yu et al., 2000; Blair et al., 2003), and evaluate intra-specific diversity (Gaitán-Solís et al., 2002). These markers were also employed to study the genetic structure in Andean and Mesoamerican races in common bean (Blair et al., 2006; Diaz and Blair, 2006; Kwak and Gepts, 2009) and population structure in 192 landraces from Ethiopia and Kenya (Asfaw et al., 2009). The use of SSRs to characterize common bean germplasm in this study is justified by their high informativeness, co-dominance, and wide distribution in the genome.

The National Crops Resources Research Institute (NaCRRI) at Namulonge holds 320 distinct accessions of common bean that are morphologically distinct but have little information documented on their genetic diversity and genetic potential. This hinders the utilization of these materials as parental sources in the different breeding programs and slows the process of designing appropriate conservation strategies. The continued adoption of elite bean varieties by farmers over time has replaced some landraces that are not continuously planted in the farmers' fields (Sekabembe, 2010) leading to genetic erosion. This erosion reduces alleles and genotype frequencies from the breeders' gene pool, narrowing variation and, hence, restricting the amount of adapted genetic diversity for future breeding. For example, a previous survey conducted in South-western Uganda, a region popular for large-seeded bean production (CIAT, 2005), found that 40% of farmers had stopped growing medium and large-seeded landraces in favor of newly introduced, small-seeded varieties that are tolerant to root-rot disease.

Pest and diseases also cause loss of bean cultivars in farmer's fields (Mukankusi, 2008). Genetic diversity is necessary for the rapid genetic improvement of crop species (Trethowan and Kazi, 2008) and its studies provide a major step towards enhancing the genetic potential of the bean germplasm. Thus, the objectives of the study was to genotype and determine the level of population structuring of common bean in Uganda using microsatellite markers for use in present and future bean breeding schemes and conservation.

MATERIALS AND METHODS

Plant material

The study included 100 accessions (Appendix 1) representative of the phenotypic diversity of common bean in Uganda. The selection of the bean sample specifically relied on two traits: (i) plant type/growth habit and (ii) weights of 100 seeds of each accession. These traits have been mapped as part of the crop's domestication syndrome (Koinange et al., 1996). The sample included the released bean varieties in Uganda and the ones frequently used in breeding activities. The place of collection/origin was also considered in order to have representatives from the different agro-ecological zones in Uganda. Three bean seeds per variety were planted in plastic pots and monitored until the first trifoliolate leaf stage in the greenhouse facilities of the Department of Plant Sciences, University of California Davis (UC Davis), U.S.A.

DNA extraction, gene pool identification and genotyping

Genomic DNA was extracted from each bean accession following procedures described in Doyle and Doyle (1990). The diluted DNA samples (30 to 40 ng/µl) were subjected to Polymerase Chain Reaction (PCR) amplification. The phaseolin marker and 22 fluorescent labeled microsatellites markers labeled according to Schuelke (2000) was used in succession to determine gene pools and genotyping accessions as follows. The gene pool identification involved PCRs set up to amplify specifically a region surrounding the 15-bp tandem direct repeat of the phaseolin gene family as in Kami et al. (1995) and Burle et al. (2010). The PCR was conducted in a MJ thermocycler in a total reaction volume of 25.2 µl (containing: 18 µl double distilled water, 10X Thermopol Reaction buffer (2.5 µl), 250 µM dNTPs (2.5 µl), 1U Taq DNA polymerase (0.2 µL), 10 µM phaseolin primer (1 µl), and 50 ng/µl DNA (1 µl). The PCR products with loading dye were loaded in a 10% vertical polyacrylamide gel electrophoresis system using 0.5X TBE buffer, and run at 130 V for 2 h. Gene pool control genotypes (BAT93 for Mesoamerican and Jalo EEP558 for Andean) were always loaded with samples. Gels were stained in 10 µl in 10 mg/ml of ethidium bromide solution and photographed for future reference. The genotypes were quantitatively assigned to either Andean or Mesoamerican gene pool based on similarity of their band patterns to the gene pool controls (Plate 1).

For microsatellite genotyping, two markers representing each linkage group were selected (Table 1) based on high polymorphism exhibited in common bean (Burle et al., 2010; Kwak and Gepts, 2009; Asfaw et al., 2009). Three fluorescent dyes: 6-FAM (Blue), VIC (Green), PET (Red) (Applied Biosystems Inc., USA) were attached to the 5' end of the M13 universal primer sequence (5'-TGT AAA ACG ACG GCC AGT-3'). The DNA was amplified separately for each primer pair in 96-well plates in a gradient thermal cycler (BioRad).

The PCR mix, contained in a total reaction volume of 20 µl, consisted of 1 µl of 30 ng DNA, 2.5 µl 10X PCR buffer (Roche), 2.5

*Corresponding author. E-mail: dennis.okii@gmail.com. Tel: +256782167552. Fax: +256414531641.

Table 1. Linkage group of microsatellite markers (SSRs), dye used in fluorescently labeling, SSR allele sizes and targeted sequence repeat for genotyping Common beans in Uganda.

Linkage group	Marker	Dye used	Allele size (bp)	SSR sequence
1	BM 200	PET	221	(AG)10
1	BMd10	PET	139	(GA)8
2	PVBR 107	VIC	150	(CT)16(GT)4
2	BM 156	PET	267	(CT)32
3	BM159	VIC	198	(CT)9(CA)8
3	BM197	PET	201	(GT)8
4	BM171	FAM	149	(GA)13
4	PVatgc002	FAM	144	(ATGC)4
5	BMd28	VIC	151	(GT)4
5	BM175	VIC	170	(AT)5(GA)19
6	BM187	PET	191	(CT)10(CT)14
6	BMd37	VIC	134	(AC)8
7	BM183	FAM	149	(TC)14
7	BM160	PET	211	(GA)15(GAA)5
8	BM189	FAM	114	(CT)13
8	BM211	VIC	186	(CT)16
9	PVat007	VIC	192	(AT)12
9	BM141	PET	218	(GA)29
10	GATS11B	PET	160	(CT)8
10	BMd42	VIC	149	(AT)5
11	BMd41	PET	250	(ATT)9
11	BM205b	VIC	137	(GT)11

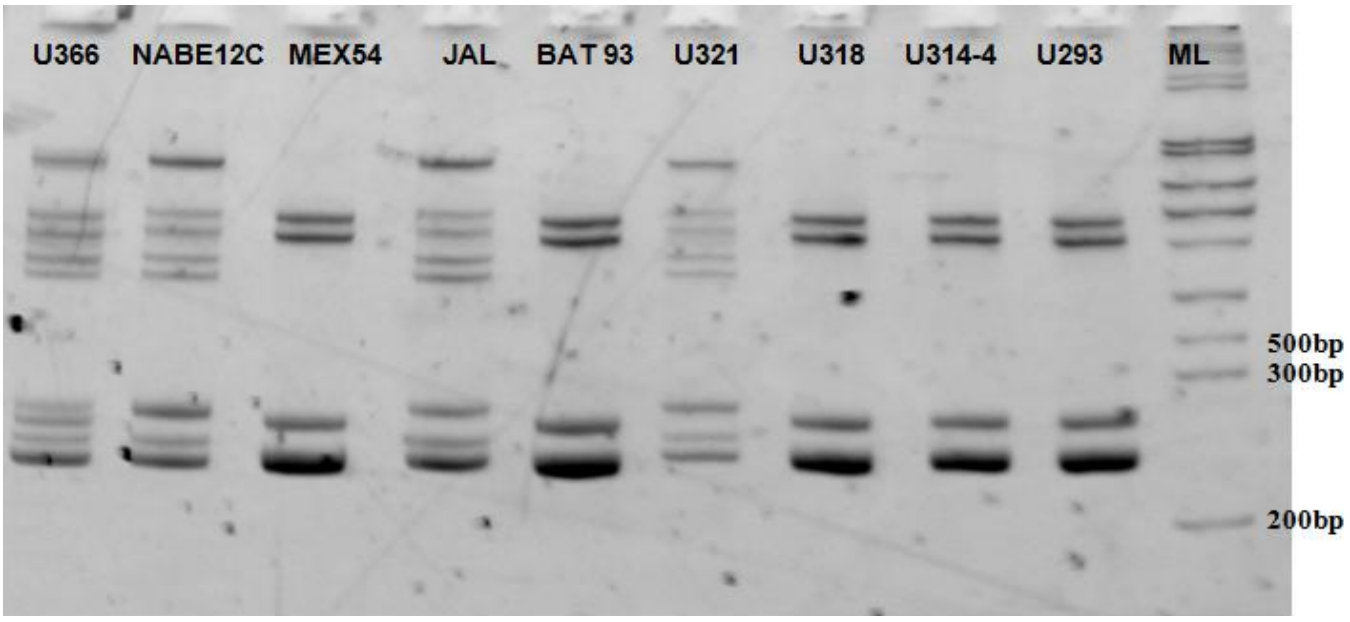

Plate 1. 10% vertical polyacrylamide gel photo showing complex band patterns used to to assign bean accessions to the Andean and Mesoamerican gene pool. ML is Hi-Lo DNA Marker ladder with size range 50 to 10,000 bp. The accessions JAL (JaloEEP558) and BAT93 are Andean and Mesoamerican control genotypes, respectively.

mmol each of the forward and reverse primers, 2 mmol dNTPs mix, 1 unit Taq DNA polymerase (Roche) and 2.5 µl of double distilled water. The PCR program had an initial denaturing step of 94°C for 5 min followed by 34 cycles of 94° for 1 min, 56°C annealing for 1 min (same temperature for all markers) and 72°C for 1 min and a further primer extension at 72° for 20 min and 4°C infinite hold.

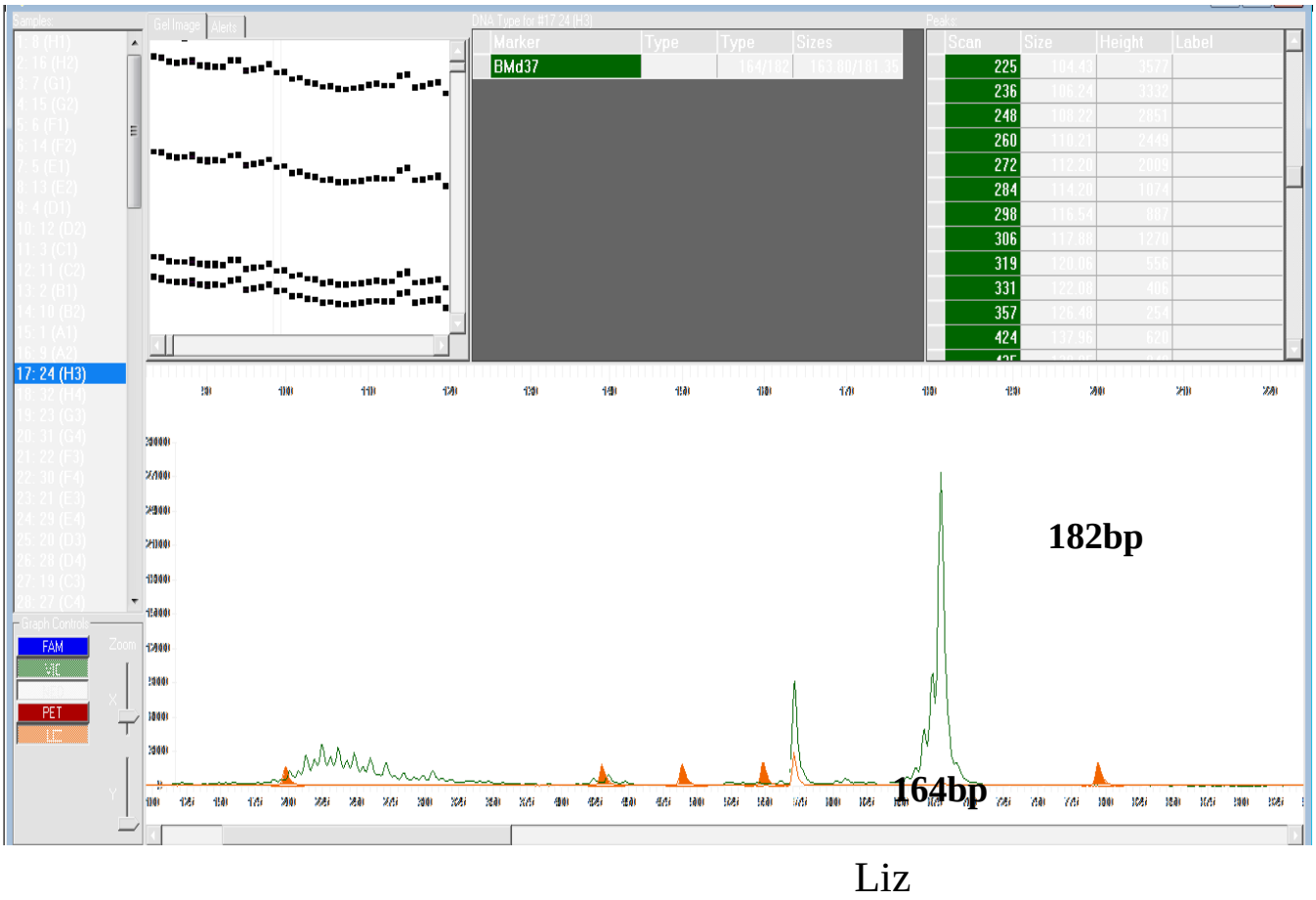

Figure 1. An electrophoregram genotype plot of the Strand 2.2.30 software, showing allele binning in sample 17:24(H3). In this case the most polymorphic marker from the study (BMd37) had two heterozygous alleles (peaks) with sizes 164/182 bp against the standard LIZ (orange) dye in the lower panel.

Prior to allele visualization by automated capillary electrophoresis, eight PCR products was randomly picked for each marker and run on 2% agarose gels to check for amplification.

Capillary electrophoresis

The preparations of PCR products for capillary electrophoresis involved markers co-loaded or multiplexed in non-overlapping panels based on expected allele size (http://phaseolusgenes.bioinformatics.ucdavis.edu/) labeled with different dyes. Multiplex panels had two to three markers with at least 30 bp size differences to avoid background stuttering of allele peaks for the different dyes during allele binning. A master mix of 10 µl consisting of 1 µl each diluted PCR product, 5.7 µl formamide (Hi-Di) and 0.3 µl GeneScan-500 LIZ size standard (Applied Biosystems Inc., USA) was prepared in optical 96-well MicroAmp plates (Applied Biosystems Inc, USA) for electrophoresis. Fragment separation was performed using the ABI PRISM 3730 genetic analyzer instrument (Applied Biosystems, USA) at the Veterinary Genetics lab at UC Davis.

Allele calling

The allele fragments were determined in strand 2.2.30 software (Mellissa et al., 2000) for peak detection and fragment size matching to the reference data (Figure 1). The allele sizes were auto-calculated with reference to the internal lane size standard GeneScan-500 LIZ, ranging from 35 to 500 base pairs. The allele size bins were exported to Microsoft Office (MS) Excel (2007) program for subsequent analyses.

Data analysis

The following genetic parameters: allele number and frequency, gene diversity, heterozygosity, and polymorphism information content (PIC) were calculated in PowerMarker software version 3.25 (Liu and Muse, 2005). The level of population structure among the bean accession was established by subjecting SSR allele sizes to a model-based program STRUCTURE version 2.3.3 (Pritchard, 2000). A Principal Coordinate Analysis (PCoA) was obtained using GenAlEx v6.4 software (Peakall and Smouse, 2006) and Unweighed Neighbor-joining (NJ) tree using microsatellite diversity implemented in the PowerMarker program generated with the Darwin program for displaying genetic relationships among accessions and to test the results of STRUCTURE.

Defining the population structure of common bean in Uganda

The STRUCTURE program (Pritchard and Stephens, 2000) is the

Table 2. Genetic diversity, observed heterozygosity (He) and number of alleles detected in 100 common bean genotypes.

Linkage group	Marker	Major allele frequency	Allele number	Gene diversity	He	PIC
1	BM 200	0.21	17	0.88	0.65	0.86
1	BMd10	0.51	15	0.69	0.72	0.67
2	PVBR 107	0.28	20	0.86	0.30	0.85
2	BM 156	0.23	26	0.89	0.51	0.88
3	BM159	0.38	13	0.69	0.06	0.64
3	BM197	0.22	14	0.86	0.96	0.85
4	BM171	0.51	11	0.68	0.10	0.65
4	PVatgc002	0.34	13	0.79	0.40	0.77
5	BMd28	0.3	15	0.83	0.66	0.81
5	BM175	0.25	21	0.87	0.45	0.86
6	BM187	0.49	12	0.71	0.40	0.68
6	BMd37	0.11	45	0.96	0.67	0.96
7	BM183	0.46	7	0.73	0.00	0.70
7	BM160	0.36	25	0.84	0.44	0.82
8	BM189	0.10	38	0.95	0.80	0.95
8	BM211	0.19	23	0.89	0.61	0.88
9	PVat007	0.17	22	0.91	0.22	0.90
9	BM141	0.29	16	0.85	0.09	0.84
10	GATS11B	0.26	12	0.83	0.33	0.80
10	BMd42	0.32	18	0.81	0.75	0.79
11	BMd41	0.36	16	0.81	0.57	0.80
11	BM205b	0.54	24	0.69	0.22	0.68
Mean		0.31	19	0.82	0.45	0.80

most widely used clustering software applied to detect population genetic structure using a defined number of pre set populations K, where each K is characterized by a set of allele frequencies at each locus. The analysis was run with 10 simulations per K value from K = 2 to 6 using 5,000 replicates for burn-in and for analysis 50,000 replicates. The “true” number of populations (K) was confirmed according to Evanno et al. (2005) using the STRUCTURE Harvester (Earl and Vonholdt, 2012), online (http://taylor0.biology.ucla.edu/struct_harvest/) for visualizing outputs. Microsoft Excel program was used for easy conversion of estimated membership coefficient and generating the bar plot. The analysis generated the membership coefficients of each subgroup and the most correct number of subpopulations (K) using different colours according to Rosenberg et al. (2002) with each individual with a fixed length line segment partitioned into K colored components.

K = 3 analysis

The individual membership coefficient at K = 3 from the STRUCTURE run had maximum mean probability of likelihood value of L (K) = -7658.3 which led to assignment of bean accessions to K= 3 three sub populations as K3.1, K3.2 and K3.3. Accessions with a membership coefficient 0.5 (50% ancestry limits) and above were clustered in the same group at the K= 3 level. The membership coefficient data generated using the STRUCTURE software was exported to MS Excel (2007) for visualization as graphical bar plot for membership coefficient of each accession within the three subpopulations.

RESULTS

Allelic diversity of the common bean germplasm

The key parameters used to define genetic diversity among beans from Uganda are presented in Table 2. There was a high level of polymorphism with a mean of 19 alleles per locus and a range of 7 to 45 alleles in the germplasm. The frequency of the major allele ranged from 0.1 to 0.51 with mean of 0.31. In total, the 22 markers detected 423 alleles, ranging from 114 to 267 bp in size and PIC ranging from 0.64 for BM159 to 0.95 for BMd37 with mean of 0.80. The overall mean heterozygosity was 0.45 with highest heterozygosity value of 0.96 in marker BM197.

Genetic diversity of Andean and Mesoamerican gene pools in Uganda

The common bean germplasm in Uganda comprised of 51% Andean and 49% Mesoamerican accessions based on the phaseolin analysis (Appendix 1). The mean allele number per locus was higher in the Mesoamerican group (16 alleles) than in the Andeans (13 alleles). The mean major allele frequency in the Mesoamerican gene pool

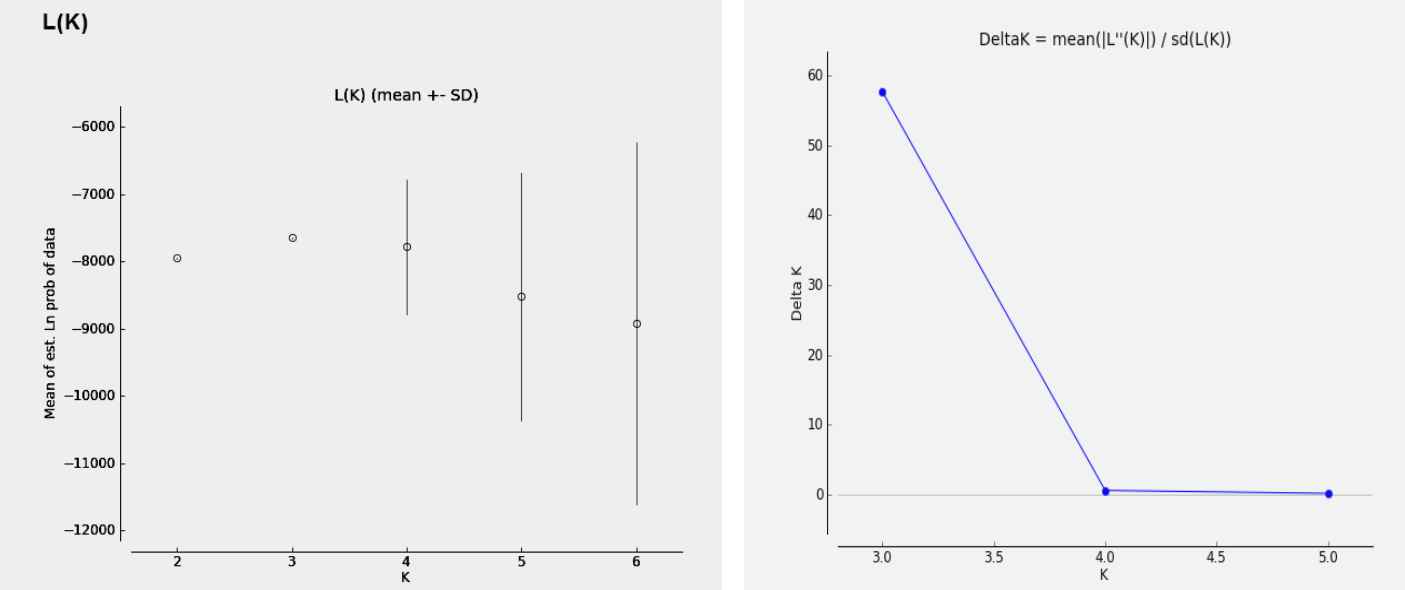

Figure 2. Plots of parameters L (K) and Delta K against the likely sub populations (K) generated according to Evanno et al. (2005) with sub-populations in Ugandan common bean with three sub populations (K=3) as most likely.

was 0.45 and 0.33 in the Andean gene pool (Table 3). The loci BMd37 and BM183 had the highest and lowest allele number respectively, among Ugandan common beans. The mean gene diversity detected was higher in the Mesoamerican (0.79) than in the Andean group (0.67). Mean heterozygosity was comparable between the Andean and Mesoamericans (0.46 and 0.44, respectively). The highest polymorphism was recorded in the Mesoamerican gene pool with a mean PIC value of 0.78 compared to 0.66 in the Andean. The most polymorphic locus in the two gene pools was BMd37, with PIC values of 0.94 among Andean and 0.96 among Mesoamerican genotypes. Locus BM189 also showed high polymerphism in the Andean materials. The markers BM183 and BM159 detected the highest allele frequency of 0.86 and 0.73 respectively in both gene pools with Mesoamerican group showing more genetic diversity.

Population structure in common bean germplasm in Uganda

The STRUCTURE clustering technique identified the population membership, structuring, and admixture as shown in the K=2 and K=3 sub-populations (Figure 3). The lowest mean probability of data was recorded at L (K) = -8382.0 for K = 6 and a highest mean probability = -7658.3 for K = 3. The Evanno test found a clear maximum for Delta K at K = 3 in the plots of L (K) versus Delta (Figure 2) confirming a likely assignment of the bean germplasm to three sub-groups. The mean genetic diversity statistics (Table 4) for the three subgroups formed at K=3 were calculated as in Hunt et al. (2011) using PowerMarker v3.25. The STRUCTURE program calculated the level of genetic differentiation or Wright fixation index of F statistics (F_{ST}) simultaneously between the different bean sub populations according to Wright (1978). The three subpopulations had moderate differentiation, with F_{ST} values ranging from 0.05 to 0.15.

Principal coordinate analysis and genotype associations

The principal coordinate analysis allowed the separation of the genotypes into three groups along the first coordinate with many cases of admixture (Figure 4). The smallest cluster comprised of Mesoamerican accessions U9-6 (95), U9-8 (97), UBR (92) 25 ml (98), VAX3 (99), VAX4 (100) and BAT93 (101), the Mesoamerican control genotype corresponds to the K3.3 sub-group in the STRUCTURE bar plot.

DISCUSSION

The objective of the study was to characterize common beans in Uganda using the phaseolin marker to determine their frequencies and membership to the Andean and Mesoamerican gene pools. A subsequent analysis with fluorescently labeled SSR markers was done to assess the levels of their genetic diversity and structure. The two gene pools of domesticated common bean are present in Uganda in similar frequencies. Previous reports show a striking difference in numbers of Andean and Mesoamerican gene pools in Africa (Bellucci et al.,

Table 3. Genetic diversity parameters in Andean (A) and Mesoamerican (M) common beans in Uganda revealed with 22 polymorphic microsatellite markers.

Linkage group	Marker	Major allele frequency		Allele no.		Gene diversity		Heterozygosity		PIC	
		A	M	A	M	A	M	A	M	A	M
1	BM 200	0.27	0.20	14	14	0.84	0.87	0.71	0.59	0.83	0.86
1	BMd10	0.58	0.45	11	15	0.61	0.71	0.67	0.76	0.57	0.68
2	PVBR 107	0.44	0.19	12	19	0.76	0.89	0.33	0.27	0.74	0.88
2	BM 156	0.24	0.34	15	22	0.85	0.80	0.80	0.22	0.84	0.78
3	BM159	0.71	0.73	8	10	0.46	0.46	0.06	0.06	0.41	0.44
3	BM197	0.35	0.30	8	12	0.77	0.81	0.94	0.98	0.74	0.78
4	BM171	0.53	0.49	9	9	0.6	0.69	0.10	0.10	0.53	0.65
4	PVatgc002	0.57	0.49	9	12	0.62	0.70	0.47	0.33	0.59	0.67
5	BMd28	0.44	0.21	11	12	0.74	0.86	0.61	0.71	0.71	0.84
5	BM175	0.48	0.33	8	20	0.7	0.84	0.53	0.37	0.66	0.82
6	BM187	0.84	0.27	8	11	0.28	0.82	0.14	0.67	0.28	0.80
6	BMd37	0.16	0.08	37	38	0.94	0.96	0.55	0.78	0.94	0.96
7	BM183	0.86	0.39	4	7	0.25	0.76	0.00	0.00	0.24	0.73
7	BM160	0.23	0.71	17	17	0.88	0.49	0.75	0.14	0.87	0.48
8	BM189	0.12	0.15	32	25	0.95	0.93	0.73	0.88	0.94	0.92
8	BM211	0.32	0.37	7	22	0.77	0.83	0.61	0.61	0.74	0.82
9	PVat007	0.34	0.20	14	17	0.78	0.89	0.18	0.25	0.75	0.88
9	BM141	0.46	0.25	12	15	0.73	0.87	0.08	0.10	0.71	0.86
10	GATS11B	0.25	0.27	9	12	0.83	0.81	0.22	0.45	0.81	0.79
10	BMd42	0.46	0.26	10	15	0.71	0.83	0.84	0.67	0.67	0.81
11	BMd41	0.29	0.43	13	10	0.8	0.74	0.65	0.49	0.78	0.71
11	BM205b	0.84	0.24	11	21	0.29	0.88	0.12	0.31	0.28	0.87
Mean		0.45	0.33	12.68	16.14	0.69	0.79	0.46	0.44	0.66	0.78

2014). Comparisons to previous studies in Eastern part of Africa, shows a gradient of the two gene pools, for example as you move from Malawi further North to Ethiopia, the proportion of Mesoamerican beans increases. In the southern part, that is, Malawi and Tanzania, Andean predominates (Mkandawire et al., 2004). In Kenya, Andeans dominate while in Ethiopia, Mesoamericans are more frequent (Asfaw et al., 2009). The great lakes region of Central Africa e.g Rwanda and Democratic Republic of Congo is predominated by the Mesoamerican gene pool (Blair et al., 2010). The suitability of markers for multiplexing, informativeness and efficiency in finding the levels of genetic diversity and structure of the different genetic groups was tested in this study. Data about genome-wide genetic variability was obtained quickly and accurately using a set of nine panels of multiplexed SSR markers that are well distributed throughout the genome and were scored semi-automatically. Ramachandran et al. (2003) also reported that capillary electrophoresis in combination with the use of fluorescently labeled primers provide high detection sensitivity of amplified DNA fragments. The total numbers of alleles were 423 with a high mean of 19 alleles per locus compared to other studies in common beans. Asfaw et al. (2009) found 389 alleles across 38 fluorescently labeled markers with a mean of 10 alleles per locus in 192 East African bean collections. Blair et al. (2010) also found 301 alleles with an average of 10 alleles per locus using 30 fluorescently labeled SSR markers from a collection of 365 common bean genotypes from Central Africa. Burle et al. (2010) reported a mean of seven alleles across 67 SSR loci in 279 common bean landraces from Brazil.

Gomez et al. (2004) found 5.7 alleles per locus in 108 small seeded individual beans from nine different sites in Nicaragua. The marked difference in frequencies of alleles recorded in this study and other studies in common bean can be attributed to differences in the number of polymorphic markers used, sample sizes, collection sites, and the geographical coverage. Common bean samples in this study included beans with different pedigrees, such as CIAT breeding lines currently used as sources of disease resistance, improved varieties such as NABE4, NABE 12C, NABE 13, NABE 14, K20, and K132, and predominantly landraces collected from farmers' fields. The high number of alleles detected in this study originates from the 22 SSRs, chosen deliberately because of their high PIC values with a high number of alleles.

Population structure refers to sub-divisions of a simple

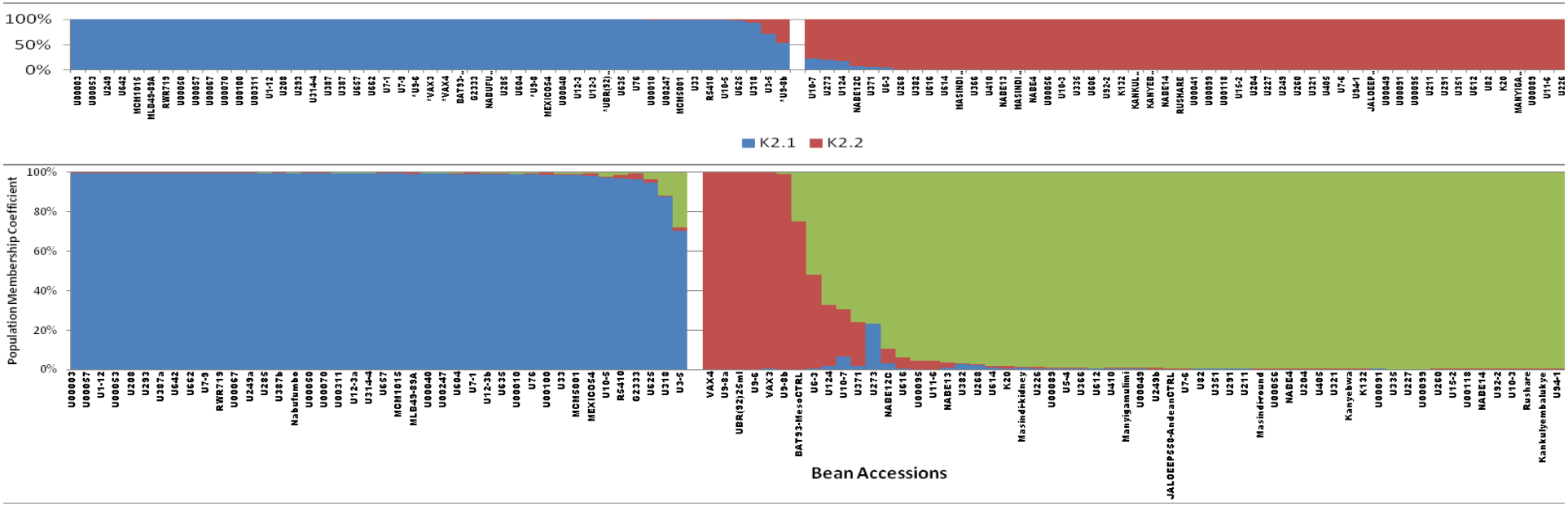

Figure 3. Hierarchical organization of genetic relatedness of 102 common bean accessions based on 22 SSRs markers from STRUCTURE program analysis described in data analysis. K=2 (above) and K = 3 (below); K3.1 K3.2 K3.3).

population in some way into smaller groups resulting from a single population's deviation from Hardy-Weinberg proportions (Falush et al., 2003a) by inbreeding, selection or migration. The modeled population structure of beans in this collection was represented graphically for K= 3 and K=2 (Figure 3). Given the existence of two major gene pools in common bean, a K = 2 situation could be reasonably predicted (Gepts and Bliss, 1985; Singh et al., 1991a). The K= 2 level consisted of sub-populations K2.1 (blue) group contained the Mesoamerican control genotype BAT93, hence, this group is included in the Mesoamerican gene pool. The second group (maroon K2.2) included control genotype JaloEEP558, a typical Andean line; hence, this group corresponds to the Andean gene pool. The STRUCTURE analysis at K=3 identified group K3.1 (blue) with (100%) Mesoamerican landraces (identified with phaseolin analysis), some of which are sources of disease resistance for bean breeding, such as RWR719 (*Pythium* root rot; Mukankusi, 2008), MEX54 (Angular leaf spot; Namayanja et al., 2006), and G2333 (Anthracnose; Nkalubo et al., 2009). Group K3.3 (green) was also identified with predominantly Andean landraces (94%). The third smaller sub-group K3.2 (maroon), identified consisted of 67% breeding lines of Mesoamerican origin such as VAX3, VAX4, and BAT93. Some accessions showed shared population membership (among K3.1, K3.2 and K3.3) and reflect the effect of hybridization or gene flow. For example, in the K3.1, accessions U33, MCM5001, MEXICO54, U10-5, R5410, G2333, U625, U318 and U3-5 were hybrids. Among the sub group K3.3 accessions, U268, U382, NABE12C, U273, U371, U10-7 and U124, also consisted of contributions from the three sub-groups identified at K=3. Some accessions derived ancestry from two subgroups, for example, K3.2 and K3.3 with accessions U616, U6-3 and BAT93 (Mesoamerican gene pool control genotype) as shown in Figure 3.

Attempts to infer gene pool of different genotypes involved relating the sub-populations members to the known gene pool controls JaloEEP558-andeanCTRL (for Andeans) and BAT93-mesoCTRL (for Mesoamericans). The ancestry of

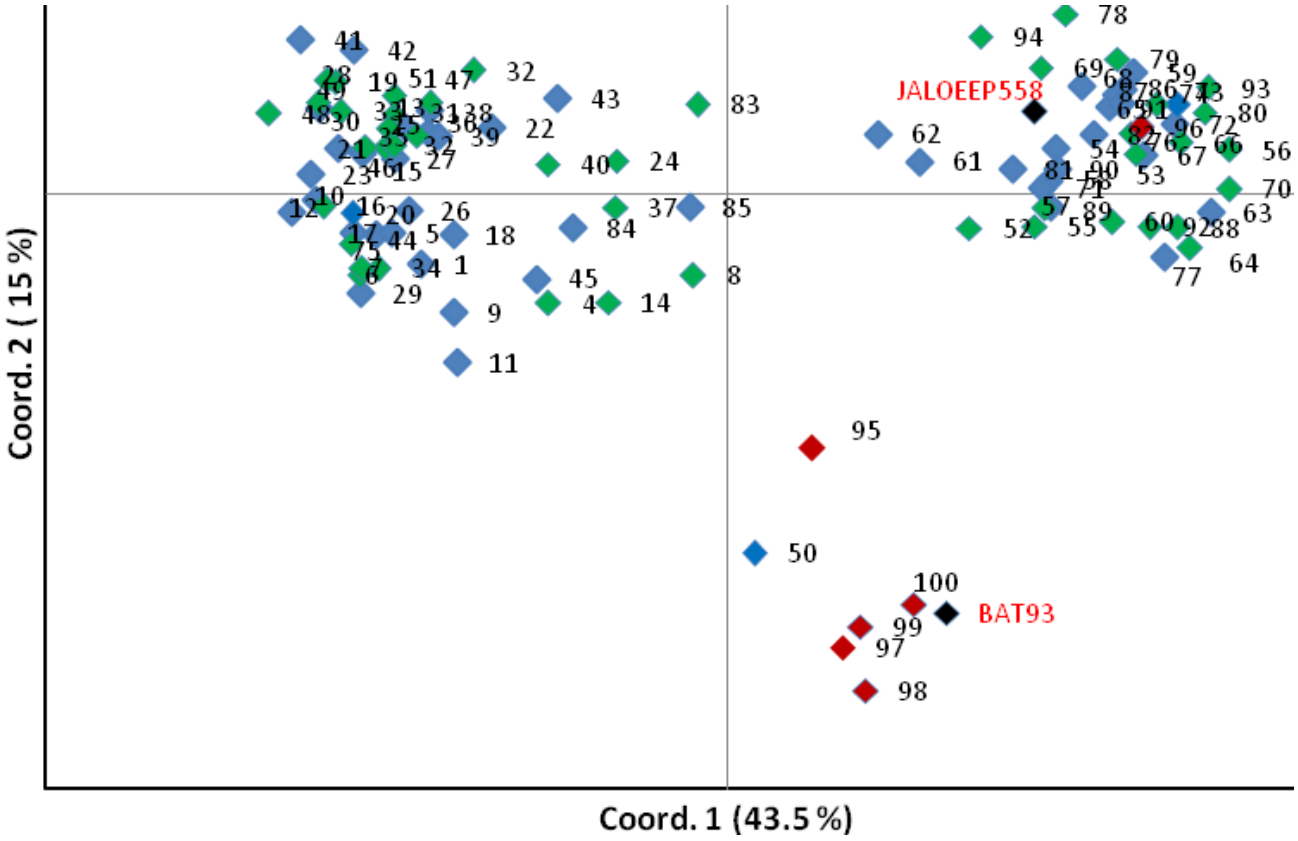

Figure 4. Principal coordinates analysis (PCoA) of accessions from microsatellites diversity based on the presence and absence of alleles. The three subpopulations are represented by diamond symbols, whose colour's reconcile with the three subgroups in STRUCTURE (Figure 3): K3.1 (blue), K3.2 (green), and K3.3 (maroon). The two black diamonds are controls, that is, genotypes JaloEEP558 and BAT93 for the Andean and Mesoamerican gene pools, respectively.

the Mesoamerican control genotype (BAT93-mesoCTRL) was shared between K3.1 and K3.2 (26% - green and 74% - maroon). Burle et al. (2010) used the same accession as a control for the Mesoamerican gene pool and reported this accession as a breeding genotype, which originates from a four-way cross, with all parents having Mesoamerican origin, hence explains its shared membership between two sub-population at K=3. The sharing of ancestry between genotypes is generally explained by recombination in some parts of the genome due to inter and intra gene pool crossings in breeding or natural hybridization. Membership switching is demonstrated by the PCoA (Figure 4) and NJ tree (Figure 5) with no correspondence between the SSR and phaseolin marker groupings, with a lot of gene pool membership switching. Membership switching among presumed sub-populations in common beans occurred in previous studies, involving allozymes and RAPD markers (Freyre et al., 1996) for presumed ancestral group of the Andean and Mesoamerican gene pools. According to Kwak and Gepts (2009), the lack of phaseolin polymorphism in domesticated gene pools prevents the detection of more subtle genetic differentiation between closely related accessions at this single albeit complex locus. The same reason can be extended to this study, in addition to admixture, to explain the lack of concordance between phaseolin and the model-based approaches used to group the bean germplasm. The long cohabitation of the two cultivated gene pools possibly led to the introgression of alleles between cultivars creating hybrids with shared phenotypic traits. The identification of hybrids in similar studies was based on the intermediate position between gene pools in the NJ trees (Bellucci et al., 2014). The high levels of admixture observed within the sub-populations in the STRUCTURE, PCoA and NJ tree analyses, clearly shows that the common bean germplasm in Uganda have considerable variations for utilizations in breeding. Model-based analyses of population divisions can be performed separately in Andean and Mesoamerican, in addition to analysing the entire sample, to detect accessions membership switching, since a marked reduction in genetic differentiation is observed by analyzing separate gene pools (Kwak and Gepts, 2009).

The STRUCTURE analysis from Burle et al. (2010) found five groups in common beans landraces from Brazil

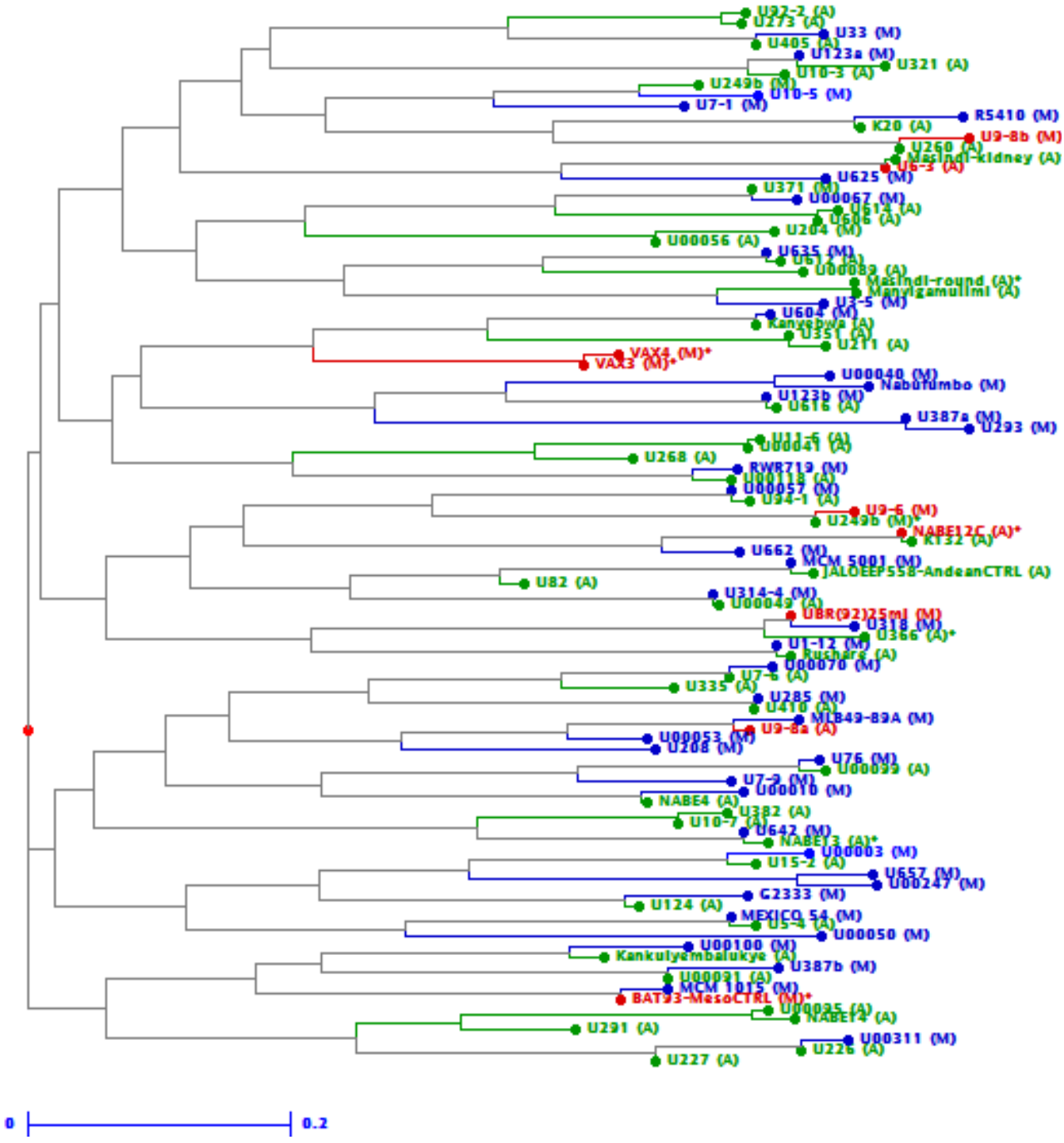

Figure 5. Un-weighed Neighbor-joining tree generated in Darwin program using microsatellite diversity of common bean accessions in Uganda, based on the Chord distance implemented in the PowerMarker program. Each branch is color-coded according to membership into the K=3 groups identified by STRUCTURE (same colors as in Figure 3). The names of accessions in brackets show their gene pool; (A) Andean and (M) Mesoamerican as determined with the Phaseolin marker, in methods and Plate 1. All the accessions in the blue branch are Mesoamerican, 95% of the green branch is Andean and 67% of maroon branch contains Mesoamerican accessions.

with strong genetic differentiation for the Mesoamerican gene pool and limited admixture between gene pools. They used 67 microsatellite markers spread over the 11 linkage groups of crop's genome and Mesoamericans

four times more frequent than the Andean gene pool. In other crops, for example, African rice (*Oryza glaberrima)*, Khady et al. (2011) identified three distinct populations in 74 rice varieties collected from Benin through population structure analysis. The high diversity in the Mesoamerican gene pool (both maroon and green components in STRUCTURE bar plot), compared to Andean in this study is due to farmers' preference for small seeded beans. In practice, preference for Mesoamerican bean types by some farmers (Blair et al., 2010) results in planting of many smaller weight seeds than larger seed weights. CIAT (2005) reported farmer's preference for smaller-seeded Mesoamerican genotypes to manage root rot disease as is the case in South-western Uganda, the leading common bean producing area in Uganda. These bean materials eventually find their ways to other parts of the country through various routes and activities.

The PCoA analysis graphical display showed that the Mesoamerican group was the most diverse and included many presumed hybrids. This observation shows that large variations arose from gene introgressions in breeding material and out-crosses in landraces that occur in farmers' fields. In other studies, Blair et al. (2010) conducted a PCoA and found diversity within and between gene pools in a larger collection of 365 common bean genotype from Central Africa. Introgression between gene pools was observed for 32 intermediate genotypes. Maciel et al. (2003) however, found no clear distinction between domesticated common bean samples from Brazil using AFLP markers and suggested that admixture was the possible cause. The common bean germplasm in Uganda has a moderate level of population structuring with F_{ST} values ranging from 0.09 to 0.14 for the three clusters generated from STRUCTURE analysis. The K3.3 group was the most differentiated with F_{ST} values of 0.14. The K3.1 and K3.2 groups followed with F_{ST} values of 0.12 and 0.09, respectively.

In other studies, Asfaw et al. (2009) found diversity in East African bean landraces and cultivars of Andean origin to be more differentiated (F_{ST}=0.331) than ones in the Mesoamerican gene pool F_{ST}=0.04) with mean F_{ST} of 0.27 among pairs of populations analyzed. They related genetic divergence in East African bean landraces to the original differences in introduced germplasm from the primary centre of origin combined with spontaneous out-crossing in farmers' fields and further farmer selection for adaptation and production uses. Further subdivision of the two gene pool into eco-geographic races (Singh et al., 1991a) was not carried out in this study and thus recommended in future to facilitate the use of the germplasm in breeding.

Conclusions and recommendations

The fluorescently labeled SSR markers revealed genetic diversity and levels of population structure within the Andean and Mesoamerican common bean in Uganda. The Mesoamerican gene pool was both more structured and genetically diverse. Gene introgressions between the two gene pools have occurred in Uganda with some accessions existing as hybrid genotypes of Andean and Mesoamerican gene pools. The 22 SSR markers used in this study showed high polymorphism among common bean samples in Uganda. Recombining Andean and Mesoamerican genes in same background could generate hybrid genotypes with broader and more durable diseases resistance for the current and future breeding programs. The common bean samples studied appears to be of great importance for breeding in Uganda and regionally as it may overcome some of the difficulties in transferring traits between the two gene pools. The bean genotypes could further be evaluated in multilocation trials to screen for agronomic traits, adaptability and biotic constraints. Regular follow up of these germplasm in the farmer's fields is vital to broaden the common bean genetic base and guard against possible losses of useful segregating phenotypes in farmer's fields. The conservation of the superior landraces can be done at NaCRRI through field regenerations and duplicated at the Plant Genetic Resources Centre (PGRC) at Entebbe in Uganda, for long-term storage.

Conflict of Interests

The author(s) declare no conflict of interests.

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Appendix 1. Gene pool and morphology of 100 common bean accessions from Uganda studied at 22 fluorescently labeled microsatellite loci.

Accession and status in breeding	Plant type	Gene pool	100 Seeds weight (g)	Seed colour	Place of collection
K132[I]	I	A	50.1	Maroon mottled	C
K20[I]	I	A	33.8	Red mottled	C
Kankulyembalukye[L]	II	A	37.2	Pink mottled	W
KANYEBWA[L]	I	A	33	Red mottled	C
MANYIGAMULIMI[L]	II	A	39.3	Red mottled	C
MASINDI ROUND[L]	I	A	35.6	Yellow	C
MASIND[I] KIDNEY[L]	I	A	31.9	Yellow	C
NABE12C[L]	IV	A	38.8	Cream specked	CIAT
NABE13[L]	I	A	41.5	Red mottled	C
NABE14[I]	I	A	42.5	Red mottled	C
NABE4[I]	III	A	40	Red mottled	C
RUSHARE[L]	II	A	42.9	Purple	SW
U00041[L]	I	A	46.8	Maroon	WN
U00049[L]	IV	A	30.8	Maroon	WN
U00056[L]	II	A	36.8	Red	WN
U00089[L]	II	A	35.3	Purple	W (Bushenyi)
U00091[L]	I	A	39.9	Red	E (Iganga)
U00095[L]	I	A	41.2	Red	E (Iganga)
U00099[L]	I	A	37.3	Red	E (Iganga)
U00118[L]	IV	A	28.8	Yellow	W (Mbarara)
U10-3[L]	II	A	50.9	Greenish-yellow	E (Mbale)
U10-7[L]	III	A	35.9	Red	E (Mbale)
U11-6[L]	II	A	43.8	Purple	WN (Nebbi)
U124[L]	III	A	32.4	Greenish-yellow	WN
U15-2[L]	II	A	33.9	Mixed	WN (Arua)
U211[L]	II	A	32.9	Mixed	WN (Arua)
U226[L]	II	A	41.9	Purple	WN (Arua)
U227[L]	I	A	44.5	Red	WN (Arua)
U249a[L]	I	A	21.7	White	SW (Kabale)
U260[L]	II	A	36.4	Red	SW (Kabale)
U268[L]	II	A	36.3	Brown	SW (Kabale)
U273[L]	II	A	43.2	Maroon	SW (Kabale)
U291[L]	II	A	44	Black	WN (Arua)
U321[L]	III	A	33	Black	WN (Arua)
U335[L]	II	A	44.4	Purple	WN (Arua)
U351[L]	I	A	40.3	Maroon	WN (Arua)
U382[L]	IV	A	40	Yellow	W (Mbarara)
U405[L]	II	A	40.8	Maroon	E (Iganga)
U410[L]	I	A	32.2	Yellow	C (Mubende)
U5-4[L]	II	A	33.5	Brown	W (Masindi)
U606[L]	I	A	42.2	Black	N
U612[L]	II	A	34.7	Red	WN
U614[L]	II	A	40.7	Red	CT (Mukono)
U616[L]	I	A	49.9	Black	NE (Kapchorwa)
U6-3[L]	IV	A	43	Red	E (Iganga)
U7-6[L]	I	A	42	Purple	WN Nebbi)
U82[L]	I	A	43.6	Maroon	CT(Mukono)
U92-2[L]	II	A	42.9	Purple	W (Mbarara)
U94-1[L]	I	A	35.2	Maroon	N

Appendix 1. Contd.

Accession and status in breeding	Plant type	Gene pool	100 Seeds weight (g)	Seed colour	Place of collection
U9-8a[L]	I	A	38.9	Maroon	W (Mbarara)
U366[L]	IV	A	36.6	Maroon	SW (Kabale)
Jalo EEP558[C]		A	35.6	Brown	Brazil
G2333[C]	IV	M	24.4	Red	CIAT
MCM 1015[C]	II	M	18.5	Cream	CIAT
MCM 5001[C]	II	M	19.5	Cream	CIAT
MEXICO 54[C]	III	M	32.4	Purple	CIAT
MLB49-89A[C]	II	M	31.2	Black	CIAT
NABUFUMBO[L]	III	M	24.8	Pink	C
R5U410[L]	II	M	18.8	Black	Rwanda
RWR719[L]	II	M	18.1	Red	CIAT
U00003[L]	III	M	15.2	White	WN (Nebbi)
U00010[L]	IV	M	21.8	White	WN (Nebbi)
U00040[L]	I	M	16.7	White	WN (Nebbi)
U00050[L]	IV	M	22	Red	WN (Nebbi)
U00053[L]	IV	M	25.8	Maroon	WN (Nebbi)
U00057[L]	III	M	22.7	Pink	W (kabale)
U00067[L]	III	M	37.2	Mixed	E (Kamuli)
U00070[L]	I	M	18	Cream	W (Bushenyi)
U00100[L]	IV	M	20.5	White	N (Lira)
U00247[L]	II	M	16.6	Mixed	E (Kapchorwa)
U00311[L]	II	M	17.9	Cream	E (Kapchorwa)
U10-5[L]	III	M	31.2	Black	E (Mbale)
U1-12[L]	IV	M	27.4	Red	W (Kisoro)
U123a[L]	III	M	21.8	Mixed	N
U123b[L]	III	M	21.9	Mixed	WN(Arua)
U204[L]	III	M	44.4	Black	E(Iganga)
U208[L]	III	M	37.7	Cream	WN(Nebbi)
U249b[L]	III	M	32.9	Red	W(kabale)
U285[L]	II	M	35.2	Mixed	W(kabale)
U293[L]	III	M	33.3	Cream	C(Mubende)
U314-4[L]	III	M	31.5	Cream	W(Mbarara)
U318[L]	II	M	16.9	Mixed	WN(Nebbi)
U33[L]	I	M	18.5	Mixed	WN
U3-5[L]	III	M	15	Mixed	W(Masindi)
U371[L]	III	M	34	Maroon	CT(Mubende)
U387a[L]	III	M	26.7	Mixture	WN(Nebbi)
U387b[L]	IV	M	29.1	Red	W(kabale)
U604[L]	III	M	24.5	Red	W(kabale)
U625[L]	I	M	18.3	White	E(Kapchorwa)
U635[L]	II	M	18	Cream	WN (Nebbi)
U642[L]	II	M	16.1	Mixed	WN (Nebbi)
U657[L]	III	M	20.1	White	W(Mbarara)
U662[L]	III	M	32.9	Mixed	E(Kapchorwa)
U7-1[L]	I	M	18.9	Red mottled	WN (Nebbi)
U76[L]	I	M	19.2	Mixed	WN(Nebbi)
U7-9[L]	I	M	30.9	Cream striped	WN (Nebbi)
U9-6[L]	II	M	18.8	Cream	WN (Nebbi)
U9-8b[L]	III	M	18.2	Maroon	WN (Arua)
UBR9225ML[C]	II	M	18.4	White	CIAT

Appendix 1. Contd.

Accession and status in breeding	Plant type	Gene pool	100 Seeds weight (g)	Seed colour	Place of collection
VAX3^{C}	II	M	21.9	Red	CIAT
VAX4^{C}	II	M	20.8	Cream	CIAT
BAT93^{C}	II	M	20.5	Cream	Brazil

Accession name: superscript is status in breeding: I=improved, L=landrace, C=CIAT line. Gene pool: A= Andean and M = Mesoamerican determined as described in the methods with the corresponding weights of 100 seeds. **Gene pool controls** are accessions: No.52 (for Andean) and 102 (for Mesoamerican). **Plant types:** I = Determinate growth habit, II = Indeterminate growth habit, III= Indeterminate prostrate growth with some climbing ability and IV= Indeterminate growth habit with strong climbing ability. **Place of collection:** Region with district name in parentheses (C = Central, W = Western, WN = West Nile, E = Eastern and N = Northern) and CIAT (regional headquarters in Uganda). **Seed coat colour:** mixed are accessions that segregated with seeds of multiple colours.

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Genetic structure and diversity of East African taro [*Colocasia esculenta* (L.) Schott]

Mercy Wairimu Macharia[1,3]*, Steven Maina Runo[1], Alice Njeri Muchugi[1] and Valerie Palapala[2]

[1]Department of Biochemistry and Biotechnology, Kenyatta University, P.O. Box 43844-00100, Nairobi, Kenya.
[2]Department of Biological Sciences, Masinde Muliro University of Science and Technology, P.O. Box 190-50100, Nairobi, Kenya.
[3]Biosciences East and Central Africa-International Livestock Research Institute, Hub, P.O. Box 30709-00100, Nairobi, Kenya.

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Taro [*Colocasia esculenta* (L) Schott] is mainly produced in Africa by small holder farmers and plays an important role in the livelihood of millions of poor people in less developed countries. The genetic diversity of East African taro has not been determined. This study utilizes six microsatellite primers to analyze five populations of taro from three different regions of East Africa. Plant material consisted of 98 taro cultivars obtained from East Africa (Kenya, Tanzania and Uganda). Principal component analysis of microsatellite data indicated variation but did not show any distinct structure. Population diversity estimate was relatively low with the highest being 0.27, for accessions sourced from Lake Victoria basin. Analysis of molecular variance (AMOVA) revealed most variation among individuals within population at 79%. Nei's genetic distance showed that relatedness is not based on geographical proximity. Based on these findings, this study proposes establishment of a regional collection that will be conserved and ensure a broad genetic base for available varieties and enable development of improved varieties through breeding programmes.

Key words: Genetic diversity, simple sequence repeats (SSRs), taro.

INTRODUCTION

Taro, *Colocasia esculenta* (L.) Schott is a member of the plant family Araceae. It is one of the most important food crops worldwide. The family comprises at least 100 genera and more than 1500 species (Cho et al., 2007). Taro is a traditional root crop of the tropics grown for its edible corms and leaves, and is believed to be one of the earliest cultivated root crops in the world (Plucknett, 1976). Worldwide production is on the increase, with Food and Agriculture Organization (FAO) records indicating that taro production has doubled over the past decade (FAOSTAT, 2000), and it is now the fifth most-consumed root vegetable worldwide. Cultivated types are mostly diploid ($2n = 2x = 28$), although some triploids are also found ($2n = 3x = 42$) (Singh et al., 2007). Taro plays an important role in the livelihood of millions of relatively poor people in less developed countries. Taro leaves and corms are used for human food in most producing countries. Its peels and wastes are fed to domestic

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

Table 1. SSR primers used in the analysis of taro (*colocasia esculenta*) collected from East Africa regions.

Primer	Repeat type	Sequence(5'-3')
Xqutem110	(TGA)6(TGGA)4	AGCCACGACACTCAACTATC GCCCAGTATATCTTGCATCTCC
Xqutem73	(CT)15	ATGCCAATGGAGGATGGCAG CGTCTAGCTTAGGACAACATGC
Xqutem55	(CAC)5	CTTTTGTGACATTTGTGGAGC CAATAATGGTGGTGGAAGTGG
Xqutem88	(CAT)9	CACACATACCCACATACACG CCAGGCTCTAATGATGATGATG
Xqutem97	(CA)8	GTAATCTATTCAACCCCCCTTC TCAACCTTCTCCATCAGTCC
Xqutem91	(TG)6(GA)4	GTCCAGTGTAGAGAAAAACCAG CACAACCAAACATACGGAAAC

livestock. Efforts have been made to produce silage from the large quantities of taro tops which are left after the corms are harvested (Kuruvilla and Singh, 1981). Taro corms are highly priced in urban markets hence generates extra income to the rural farmers while its trade provides employment for many people. The crop also maintains ground cover in the fields (Tumuhimbise et al., 2009). However, there is very limited local research on taro in East Africa and its actual contribution to food security and economy is underestimated. Also, its profile on the national research and conservation agenda is low. In Uganda for example, the average taro yields is less than 1 t ha^{-1}. The majority of smallholder produces annually (Tumuhimbise et al., 2009) as compared to the African and world average of 5.9 and 6.6 t ha^{-1}, respectively (FAO, 2008). It is possible to increase the status of taro in east Africa to the level of potato and sweet potato. This can be done by diversifying the taro germplam grown in East Africa including the dryland (unflooded) or upland taro varieties.

Morphological characters in tubers are highly variable among the genotypes. This high variability is a result of natural mutations for traits like root and skin color, leaf and vine characteristics (Karuri et al., 2009). However morphological variability may be as a result of environmental changes and therefore it is important to carry out a genetic diversity study using molecular markers. This study aims to quantify the level of genetic diversity and distribution of taro germplasm within the East African region SSR molecular markers. This information is crucial in formulating breeding programs aimed in improving the quality of taro germplasm in East Africa.

MATERIALS AND METHODS

Plant materials and DNA extraction

Plant materials consisted of taro cultivars obtained from Lake Victoria basin that covers three countries: Kenya, Tanzania and Uganda. Additional samples from Central and Eastern Kenya were incorporated in the study. The different cultivars were collected from farmers. Collections involved both principal botanical varieties of taro - *Colocasia esculenta* var. *esculenta*, commonly known as dasheen, and *Colocasia esculenta* var. *antiquorum*, commonly known as eddoe. The planting material was collected as apices, 1-2 cm of the corm with the basal 15-20 cm of the petioles attached; use of apices as planting material is particularly advantageous because it does not entail the utilization of much material that is otherwise edible. Moreover, apices establish very quickly and result in vigorous plants. The apices attached to part of the corms were planted in pots in the green house and appropriately labeled. The plants were watered every day to ensure growth, after a month, young shoots had already formed. DNA was extracted from young leaves according to the CTAB protocol modified and optimised by Sharma et al. (2008).

PCR amplification

The present study used six SSR primers (Table 1) that revealed high level of polymorphism in Polynesian taro cultivars (Mace and Godwin, 2002). The PCR mixture (10 µl) contained 1 µl of template DNA, 0.05 µl forward primer and 0.05 µl reverse primer, 0.05 µl of each dNTP (A, T, C, and G), 1 µl of reaction buffer that contained magnesium chloride and 0.25 µl *Taq* DNA polymerase. The PCR regime consisted of initial denaturation (94°C for 5 min), 35 cycles each consisting of 30 s denaturation (94°C), 1 min annealing temperature ranging from 55 to 59°C and 2 min elongation (72°C). Finally, an extension period of 10 min was included. A 10% native PAGE gel was used to resolve the SSR products as described by Andrus and Kuimelis (2001).

Data analysis

The number of individual bands generated by each primer was checked visually and scored as presence and absence matrix. The resulting presence/absence data matrix was analyzed using POPGENE v. 1.31 (Yeh and Yang., 1999), assuming Hardy-Weinberg equilibrium, to estimate three genetic diversity parameters: the percentage of polymorphic loci (PPL), Shannon's information index of diversity (*I*), Nei's gene diversity (*H*) and Nei's genetic distance (Nei and Li,1979). Analysis of molecular variance (AMOVA) was used to partition the total SSR variation into within-

Table 2. Mean diversity estimates (H) for 5 populations of *C. esculenta* generated from 6 SSR markers; N: sample size, H: Gene diversity and I Shannon Index.

Ecotype	N	*H*	I	Polymorphic loci (%)
Lake Victoria basin (Taro)	33	0.2783	0.4871	90.32
Lake Victoria basin (Tannia)	22	0.2478	0.4672	80.65
Central Kenya (Taro-Girigasha)	10	0.1774	0.3590	64.52
Central Kenya (Taro-Kigoi)	10	0.1261	0.2476	48.39
Eastern Kenya (Taro)	23	0.1863	0.3406	58.06

Table 3. Analysis of molecular variance (AMOVA) for SSR among *C. esculenta* sampled from Kenya, Uganda and Tanzania. Degrees of freedom (d.f) and significance (P) of the variance components, Sum of square (Ss), mean of square (Ms), and estimated variance (Est.Var) are shown.

Source of variation	df	Ss	Ms	Est.var	percentage	P-value
All population						
Among regions	2	61.083	30.541	0.357	6	0.01
Among population	4	39.088	19.544	0.824	14	0.01
Within population	93	422.676	4.545	4.545	79	0.01
Total	99	522.847		5.726	99	

population and between-population (Excoffierer et al., 2005). Principal coordinate analysis was done using GenAlEx 6.1 this is a multivariate technique that allows one to find and plot the major patterns within a multivariate data set (Peakall and Smouse, 2001).

RESULTS

In this study, six primers developed by Mace and Godwin (2002) were used and showed different levels of polymorphism. The six primers used differed in the ability to identify unique multiband phenotypes among the 98 accessions. In total, 31 alleles were amplified from six SSR primers across 98 accessions of which 85% were polymorphic.

No region specific markers were amplified. The gene diversity values (H) ranged from 0.2783 for Lake Victoria basin (taro) to 0.1863 for Eastern Kenya (taro) as shown in Table 2. The highest proportion of percentage polymorphic loci was found in taro from Lake Victoria basin while the lowest was found in Central Kenyan taro (Kigoi variety). Girigasha variety from Central Kenya had 64% of polymorphic loci while taro from Eastern Kenya had 58.06% polymorphic loci (Table 2). A general trend between the highest % polymorphic loci and highest gene diversity was observed. The standard error for all the population was generally high (± 0.1859) in taro from private alleles across the entire collection. Accessions of taro from Lake Victoria basin were the most distinguishable based on the SSR data set.

Genetic structure

In this study, there was no significant differentiation among the populations of *C. esculenta*, using SSR markers (AMOVA, P>0.01) (Table 3). The overall genetic variation (79%) was ascribed to differences between samples within varieties. The variation among regions was 6 and 14% among populations. A plot of the first two principal components of analysis of SSR variation represented an overall variation of 32% in the first axis and 19% in the second axis (Figure 1). There was no distinguishable pattern of clustering of accessions from a certain region. Taro-Kigoi samples from central Kenya revealed little variation in the first axis. Taro-Girigasha from Central Kenya was also in the first axis. Tannia and Taro from Lake Victoria basin showed high variation across the two axes, this was also true for the Eastern Kenya taro. Genetic distance based on Nei and Li (1979) is shown in Table 4.

DISCUSSION

An understanding of the extent and distribution of genetic variation within and among taro populations is essential for determining appropriate genetic management strategies for the species. The amount of molecular marker information has considerable impact on the results of studies of germplasm genetic relationships in crop (Cheng-lai et al., 2010). In this study, a total of 31 alleles were amplified with an average of 6.1 alleles per locus, this agrees with results obtained by Mace et al. (2006) who used similar primers to rationalize taro germplasm in the Pacific; in their study, a total of 38 alleles were amplified in 515 accessions of taro. No allele was found to be specific for any population.

Average genetic diversity (H) for all the taro populations assessed was 0.20318 with the highest being taro and Tannia from Lake Victoria basin populations (H=0.2783

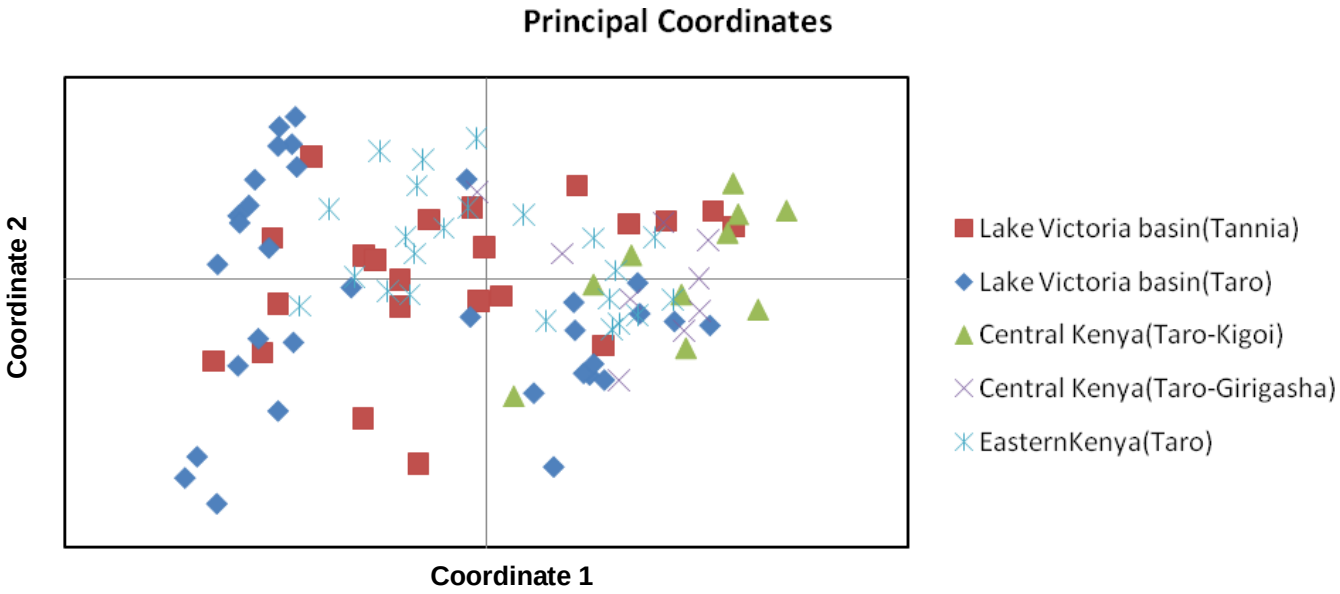

Figure 1. Distribution of 98 accessions on the first and second coordinates of PCA performed with six markers. The accessions are represented according to their geographical region.

Table 4. Matrix of unbiased genetic distance according to Nei (1978) among 5 populations of *C. esculenta* based on 6 SSR markers; where, 1- Population from Lake Victoria basin (Taro), 2- Population from Lake Victoria basin (Taro-tannia), 3- Population from Central Kenya (Taro-girigasha), 4- Population from Central Kenya (Taro-Kigoi) and 5- Population from Eastern Kenya (Taro).

Pop 1D	1	2	3	4	5
1					
2	0.0766				
3	0.2733	0.1931			
4	0.1597	0.128	0.1265		
5	0.0876	0.0371	0.1753	0.0894	

and H=0.2478, respectively). This is low as compared to genetic diversity of sweet potato in Kenya whose average was 0.75 with the lowest being 0.21 (Karuri et al., 2009). In the genetic diversity of cassava in the great lakes region, value of H was above 0.5 which was considered sufficient for a conservation program (Pariyo et al., 2009; Tumwegamire et al., 2011). The highest genetic diversity was in Lake Victoria basin, this could be attributed to the fact that the Lake Victoria basin covers three countries namely: Kenya, Uganda and Tanzania. The three countries are separated by a lake, this feature is a probable reason as the individual countries germplasm is not exchanged among farmers in the three countries.

Taro from central Kenya harbored low genetic diversity; girigasha (H=0.1774) and Kigoi (H=0.1261). These two populations consist of two different varieties. None of the accessions from Kigoi overlap with cultivars from girigasha, this is clearly shown by the PCA analysis. The low genetic diversity in the central region is attributed to clonal propagation indicating the use of planting material from a common source. It is worth noting that clonal plants have lower genetic diversity than non-clonal plants (Harper, 1977). This phenomenon was reported by Fajardo et al. (2002) and Gichuki et al. (2003), who also studied genetic diversity in the clonally propagated sweet potato. Despite these findings, Pujol et al. (2005) while studying genetic diversity of cassava emphasizes the incorporation of volunteer seedlings, of predominantly vegetatively propagated crops, by traditional farmers as an important mechanism for increasing genetic variability and a potential avenue for avoiding genetic erosion. Diversity in clonally propagated crops is attributed to accumulation of random mutations resulting via stem cuttings and adventitious buds arising from storage (Karuri

et al., 2009). Low levels of genetic diversity are detrimental to populations as they lead to inbreeding depression. However, they can be of interest in evolutionary studies as they may indicate ongoing evolution and speciation (Shepherd, 1999). The results signify the importance of assessing populations for variability for conservation purposes.

Results from AMOVA analysis showed low genetic variation among populations but high genetic variation within population. This agrees well with report of Kreike et al. (2004) and Mace et al. (2006). This may be attributed to the limited number of taro accessions introduced to populations. The accessions collected from the three countries were grouped into populations across the regions, namely: Lake Victoria basin (which covers Kenya, Uganda and Tanzania) Central Kenya and Eastern Kenya. In general, no population was found to be unique and some varieties had duplicates in the collection.

From the principle component analysis, a few accessions from the Lake Victoria basin were distinct and formed one cluster displaying divergence from other regions. Varieties from Eastern region have a higher similarity with the tannia varieties from the Lake Victoria basin as compared to varieties from central region implying that planting material must have been sourced from the same area. The genetic distances between taro from Lake Victoria basin and taro-girigasha from central Kenya was the highest (0.2733) as shown in Table 4. The smallest genetic distance was between tannia from Lake Victoria basin and taro from Eastern Kenya (0.0371) as shown in Table 4.

Overall results indicated that accessions did not cluster as dasheens or eddoes and neither did they cluster according to geographical region and cultivars known by the popular names did not always cluster together. This indicates diversity within the variety or misidentification. This study has contributed critical knowledge about the distribution of genetic variation within and among the taro germplasm collections of the East African regions. There is a narrow genetic base among and within taro cultivars in the East African taro and only the accessions from Lake Victoria basin analyzed have sufficient genetic diversity that can be used to enhance breeding and conservation.

This agrees with a study conducted by Elameen et al. (2008) on sweet potato varieties who found that only few of the populations studied had sufficient diversity. Lack of genetic diversity implies that the crop may be susceptible to pest and diseases as there is no enough material to evaluate superior varieties and thus leading to enormous loss of crop. This was the case of Samoan Archipelago in 1993, seriously damaging the crop. The severity of this epidemic was mainly due to extensive plantings of the same susceptible taro cultivar. A USDA program, Agricultural Development in the American Pacific (ADAP), financed from 1994 to 1996 the collection, evaluation and distribution of leaf blight resistant taro to American Samoa. Taro production quickly recovered, but almost 10 years later, the number of different varieties remained (TaroGen, 2000).

Conclusion

This study will further help to support the use of molecular markers for the successful development of a core collection of taro germplasm for the East African region. This study provided insights into the genetic composition of the taro crop in East African regions with Lake Victoria basin hosting the greatest diversity and Central Kenya having the lowest diversity.

Partitioning of genetic variation of the populations indicated that the species is characterized by high within population genetic diversity. The study has also demonstrated the usefulness of SSR markers in genetic diversity analysis of clonally propagated crop. SSR results showed that the sampled populations of *C. esculenta* are not significantly different. Despite concerns of genetic erosion due to clonal propagation, the accessions obtained from the same variety harbored some genetic differences. Partitioning of genetic variation of the populations indicated that the species is characterized by high within population genetic diversity.

Conflict of Interests

The author(s) declare no conflict of interests.

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Assessing relationship between phenolic compounds and resistance to *Phytophthora megakarya* using two cocoa (*Theobroma cacao*) families

SIMO, C.[1,5]*, DJOCGOUE, P. F.[1,2], MBOUOBDA, H. D.[1,3], EFFA, P. O.[1,4], BOUDJEKO, T.[4], NDIANG, Z.[5] and OMOKOLO, D. N.[1]

[1]Laboratory of Plant Physiology, Department of Biological Sciences, Higher Teacher's Training College. P. O. Box 47 Yaounde Cameroon.
[2]Department of Plant Biology, Faculty of Science, P.O. Box 812, Yaounde, Cameroon.
[3]Department of Biology, Higher Teacher Training College (HTTC), Bambili, The University of Bamenda, P. O. Box 39 Bamenda, Cameroon.
[4]Department of Biochemistry, Faculty of Science, P.O. Box 812, Yaounde, Cameroon.
[5]Department of Botany, Faculty of Science, University of Douala P. O. Box 24157, Douala, Cameroon.

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Black pod disease is an important fungal infection in cocoa (*Theobroma cacao* L.) which causes high production losses. In Cameroon, these losses reached 80% of cocoa production depending on ecological zones. In order to contribute to the efficiency of selection methods used in resistance or tolerance to black pod disease with the aim of improving on cocoa farming, the content of phenolic compounds was analyzed on the genotypes of two hybrid families (F79:♀$T_{79/467}$x♂SNK_{13} and F13:♀SNK_{13}x♂$T_{79/467}$) of cocoa which are different in productivity and vulnerability to black pod disease. After artificial inoculation of the pods by mycelium of *Phytophthora megakarya*, the content of the phenolic compounds significantly increased in all genotypes of the two families. The heterosis effect of each family revealed a higher variability within both families. These results alike showed that productive and tolerant genotypes (F1307, 1314, F7902 and F7928) have a high phenols content and positive heterosis meanwhile the less tolerant and productive genotypes (F1321, F1326, F7904 and F7911) have a weak content and negative heterosis.

Key words: Cocoa, disease, tolerance, heterosis effect, phenolic compounds, hybrid progenies.

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is a perennial crop of significant economic importance in producing countries of Africa, South America and South East Asia. Currently, over 50 countries are engaged in cocoa production and heavily rely on cocoa exportation for their economic development, as this commodity contributes significantly to foreign exchange earnings. However, the cultivation of this plant is faced with numerous problems such as

*Corresponding author. E-mail: simoclaude@yahoo.fr. Tel: (237) 77 58 92 87/91 78 36 07.

ageing of plantations, parasitic constraints, black pod disease due to by *Phytophthora megakarya*, which causes significant loses worldwide. These losses globally attained 20 to 30% of production, but might reach 80% in some Central African countries (Pokou et al., 2008; Simo et al., 2011). In Cameroon, *P. megakarya* causes over 80% losses depending on the ecological zone (Djocgoue et al., 2010) which may reach 100% if no control measures are enforced (Ndoumbe-Nkeng et al., 2004). The predominant symptom of pods is a brownish or black lesion on the husk, leading to blackening and rotting. On stems, the symptoms appear as cankers (Opoku et al., 2007; Nyadanu et al., 2012). Even though damage due to cocoa pod and leaf infections by *P. megakarya* are difficult to estimate, their effect on the health and productivity of cocoa trees are significant.

The control of black pod disease is, therefore, a major challenge for world cocoa cultivation and selection of resistant materials is underway in many producing countries. According to Tan and Tan (1990), several methods have been adopted by famers to control disease caused by *Phytophthora* species in cocoa, of which use of copper-based fungicides is the most predominant. Although this is reasonably effective, the high cost of chemical control in Africa poses a serious challenge to peasant farmers who produce over 50% of world cocoa. Moreover, the chemical control is unattractive from commercial and environmental points of view and is not always effective. It is also toxic to animals including man and, therefore, poses a great danger to peasant farmers most of whom are illiterates (Opoku et al., 2000; Nyadanu et al., 2012).

The most practical and appropriate means to control cocoa black pod is by the use of resistant or tolerant genotypes, supported by further measures of an integrated control system (Adomako, 2006; Nyasse et al., 2007). Although complete resistance has not been detected, differences in susceptibility among clones or among hybrids derived from crosses have been observed in various countries, including Cameroon (Lockwood et al., 2007; Djocgoue et al., 2010). Host plant resistance in cocoa is described as polygenic and additively inherited (Tan and Tan, 1990).

A strong correlation exists between the production and the use of hybrids due to the heterosis effect. Heterosis is the heterozygotic manifestation and this manifestation is the hybrid vigour. On the phenotypic map, heterosis effect showed the performance of the hybrid genotypes which are higher than the one of the mean of the two parents. During the development of the necrosis, positive heterosis has negative values while in the productivity and phenolic compounds we have positives values.

One of the priority objectives for cocoa farming in Cameroon is the selection of genotypes, which are less vulnerable to black pod disease and productive (Djocgoue et al., 2010). Nevertheless, the rate of cocoa pods attack ranges from one to another. Nyasse et al., (2002) have proven the existence of a correlation between cocoa resistance vis-à-vis to *P. megakarya* and the development of the lesion on cocoa pods obtained through artificial inoculation of the pathogenic agent by the lesion surrounded by living cells which have become resistant to microorganisms. Those cells have their cell walls reinforced by a layer of cellulose, lignin and other phenolic compounds (El Hassni et al., 2004; Dogbo et al., 2008). Numerous biochemical pathways are stimulated and secondary metabolisms increased. All these reactions constitute the hypersensitive response. It occurs after the regeneration of the pathogenic agent in the resistant plant. The role of phenolic compounds in plant defense is well documented (Tan et al., 2004; Omokolo and Boudjeko, 2005; Mbouobda et al., 2010). Generally, phenolics accumulate at different levels in infected tissues in response to pathogen invasion. The resistance of apple (*Malus domestica*) to *Ventura inaequalis* is related to the higher content of catechin and proanthocyanidins in leaves (Treuter and Feucht, 1990). Daayf et al. (1997) reported the accumulation of a methylester, *p*-coumaric acid, in leaves of cucumber (*Cucumis sativus*) infected by *Sphaerotheca fuliginea*. In the date palm there is a higher accumulation of non-constitutive hydroxycinnamic acid derivatives in resistant cultivars (El Hadrami et al., 1997). Some plants can induce a large variety of phenolic phytoalexins. Kodama et al. (1992) listed some16 phytoalexins produced by rice (*Oryza sativa*) in response to pathogen attack.

In the present study, the assessment of heterosis effect of the phenolic compounds in the resistance of *T. cacao* vis-à-vis to *P. megakarya* was done. The quantitative analyses of the content of phenolic compounds in the healthy pods, scarified and inoculated pods with sterilized agar disc and scarified and in inoculated pods with *P. megakarya* mycelium were also realized in function of their productivity and necrosis surface. Finally, the study of the heterosis effect of phenolic compounds vis-à-vis to *P. megakarya* from parental clones to hybrid progenies was also performed.

MATERIALS AND METHODS

Plant material

The plant material derived from the experimental station of the Cocoa Development Corporation (SODECAO) was made up of 3-months-old cocoa pods that belong to two parental clones (the sensitive and productive SNK13, Trinitario group, a tolerant and less productive T79/467, Forastero group) as well as their hybrid progenies organized within the F13 (♀SNK13x♂T79/467) and F79 (♀T79/467x♂SNK13) populations. The fungal material was a local isolate strain of *P. megakarya* (TA121) obtained from the Institute of Agricultural Research for Development (IRAD) of Nkolbisson (Yaounde, Cameroon).

Cocoa pods inoculation

The apparently healthy pods were harvested, washed with tap

Table 1. Mean surface area of necrosis (cm^2) on cocoa pods 6 days after inoculation with *Phytophthora megakarya* and productivity of cocoa beans weight (g).

Genotype	Mean surface area of necrosis (cm^2)	Mean of 100 cocoa beans weight (g)
Parents		
SNK13	59.04±3.41^{i}	406.17±12.92^{hi}
T79/467	39.62±2.23^{g}	360.50±7.71^{ef}
F13		
F1307	6.16±1.46^{ab}	450.14±37.35^{lm}
F1314	17.85±0.43^{de}	581.23±12.79^{o}
F1315	16.62±1.35^{cde}	296.77±3.75^{ab}
F1313	20.98±1.23^{ef}	304.64±14.60^{ab}
F1324	69.44±2.95^{k}	519.01±5.15^{n}
F1308	42.05±4.61^{h}	441.94±33.55^{klm}
F1321	102.13±3.58^{m}	292.18±2.16^{a}
F1326	64.12±2.17^{jk}	304.07±2.48^{ab}
Parents		
SNK13	59.04±3.41^{hi}	406.17±12.92^{gh}
T79/467	39.62±2.23^{f}	360.50±7.71^{ef}
F79		
F7902	9.94±1.79^{a}	439.32±4.92^{hi}
F7928	11.88±3.09^{ab}	409.90±26.40^{gh}
F7926	13.73±2.66^{abc}	271.28±4.14^{a}
F7907	15.6±3.14^{abc}	249.87±20.30^{a}
F7915	74.86±6.12^{j}	550.36±8.46^{l}
F7919	50.46±3.22^{gh}	500.05±50.82^{k}
F7904	59.04±3.41^{hi}	252.80±21.79^{a}
F7911	57.47±4.70^{hi}	252.69±7.84^{a}

Values followed by the same letter within column for each family are not significantly different (P< 0.05).

water, sterilized with 70% alcohol and divided into three groups. The first group consisted of healthy pod (H), second group of pods scarified and inoculated with sterilized agar disk (S), third group of pods scarified and inoculated with an agar disc containing *P. megakarya* mycelium (I) obtained from 7-day-old PDA culture medium and incubated at 25 to 26°C in the dark in a humid chamber. The measurement of the necrosis surface area was done in days 3, 4, 5 and 6 after inoculation. The diameter of the circular necrotic spots was measured and the surfaces calculated using Blaha and Lotode's formula (1976).

Assessment of productivity

The productivity was assessed by measuring the weights of 100 fresh cocoa seeds in the different genotypes (Cilas, 1991).

Determination of heterosis

Heterosis or hybrid vigour is estimated by comparing the hybrid vigour of F_1 to the mean of those of the two parents (P_1 and P_2). This hybrid vigour (HF) is calculated according to Gallais (1990) and Zahour (1992) and is expressed in percentages (%).

Genotypic identification for phenolic compounds analysis

The parental and hybrid genotypes were identified for biochemical studies such as tolerant and productive (F1307, F1314, F7902, F7928), tolerant and less productive (F1315, F1313, F7926, F7907), less tolerant and productive (F1324, F1308, F7915, F7919) and less tolerant and less productive (F1321, F1326, F7904, F7911) (Djocgoue et al., 2010) (Table 1). The study of the necrosis and the productivity also permit obtaining heterosis effect. These different groups have been selected from the morphological study to use them in the study of phenolic compounds. This step will permit to see if there is a similitude between the morphological study and the study of phenolic compounds (Tables 2 and 3).

Phenolics analyses

For the analysis of phenolic compounds, samples were taken 6 days after inoculation from healthy tissue at approximately 2 cm outside of the lesion. Total phenolic compounds were extracted twice using methanol (80%). One gram of fresh tissue was ground in 5 ml of methanol (80%). After 30 min incubation at 4°C, the ground material was centrifuged at 6000 *g* for 20 min. The supernatant was collected and the precipitate re-suspended in 3 ml of methanol and incubated at room temperature for 15 min followed by another centrifugation. The supernatant was collected and mixed with the first to constitute the phenolic extract. The concentration of phenolic compounds was determined in the supernatant spectrophotometrically at 725 nm, according to Marigo (1973), using the Folin-Ciocalteu reagent. Phenolic contents were expressed in milligram equivalent of chlorogenic acid/gram of fresh

Table 2. Heterosis value (%) with respect to average parents of the necrosis of F13 family and F79 family.

Genotype	Time (day)			
	Day 3	Day 4	Day 5	Day 6
F 13				
F1307	-100	-88.53	-85.20	-87.51
F1314	-77.89	-63.29	-75.34	-63.81
F1315	-100	-45.43	-57.60	-66.30
F1313	-81.90	-84.33	-54.43	-57.47
F1324	+17.30	+1.20	+45.38	+40.76
F1308	-11.71	-30.07	-11.94	-14.75
F1321	-4.18	+21.42	+69.667	+107.03
F1326	-61.50	-54.90	+10.11	+29.98
F 79				
F7902	-100	-86.02	-81.42	-79.84
F7928	-100	-100	-90.00	-75.91
F7926	-100	-81.05	-68.92	-72.16
F7907	-100	-100	-92.50	-68.37
F7915	-100	+6.11	+87.23	+51.75
F7919	-68.06	+19.32	+26.76	+2.29
F7904	-100	+10.41	+33.96	+19.68
F7911	-63.32	-19.27	+4.77	+16.50

Table 3. Heterosis value (%) with respect to average parents during three years of the productivity of F13 family and F79 family.

Genotype	Time (year)		
	First year	Second year	Third year
F 13			
F1307	+26.77	+22.27	+5.57
F1314	+55.33	+50.04	+53.96
F1315	-22.01	-23.30	-24.08
F1313	-23.45	-23.33	-16.63
F1324	+41.51	+32.38	+35.73
F1308	+7.94	+17.14	+21.27
F1321	-24.25	-25.23	-23.69
F1326	-22.23	-22.00	-21.06
F 79			
F7902	+15.27	+14.36	+14.17
F7928	+0.84	+10.37	+9.40
F7926	-27.89	-31.15	-28.59
F7907	-39.61	-31.25	-33.71
F7915	+48.58	+41.54	+40.70
F7919	+48.28	23.13	+20.39
F7904	-37.23	-36.95	-28.03
F7911	-35.15	-34.41	-32.69

weight.

Statistical analysis

Data from this study are presented in the form of means ± SD, for at least three independent experiments during three successive trips. Three measurements were recorded for each campaign. Analysis of variance (ANOVA) and Duncan test were used to compare the susceptibility levels and phenolic contents of better progenies resulting from different crosses to access hybrid vigour, using SPSS 12 version for Windows P value less than 0.05 was considered significant. Principal component analysis (PCA) and hierarchical classification were performed with SPAD version 4.1 windows

software to have different groups of hybrids genotypes in function of the degree of their resistance.

RESULTS

Evaluation of the phenolic content and the heterosis effect

Phenolic content

In healthy condition, the accumulation of total phenolic content is more important in parental genotype SNK13. This accumulation, though important in situation of wound or inoculation, remains less than that observed in the tolerant genotypes T79/467.

In the hybrids of F13 family, in healthy conditions, the phenolic content was more abundant for F1321 (5.57 ± 0.46 $mg.g^{-1}$ of FW), followed by F1324 (5.42 ± 0.29 $mg.g^{-1}$ of FW), F1315 (5.40 ± 0.29 $mg.g^{-1}$ of FW) and F1308 (5.13 ± 0.43 $mg.g^{-1}$ of FW). In conditions of scarified and inoculated with sterilized agar disc (S), or scarified and inoculated with *P. megakarya* mycelium (I), there was an accumulation of total phenol content in both parents and all hybrids. This accumulation is more perceptible in F1313 (109%), F1307 (71%) and F1315 (58%) hybrids in healthy condition and in F1313 (202%), F1307 (186%), F1314 (109%) and F1315 (76%) for inoculation condition. Hybrids with lower necrosis surface had lower phenol content. However, the total phenols content was more abundant in inoculated conditions than in healthy conditions (Figure1a).

Under healthy conditions in F79 family, phenols contents are more abundant in hybrids F7904 (5.80 ± 0.54 $mg.g^{-1}$ of FW) and F7911 (5.25 ± 0.33 $mg.g^{-1}$ of FW). Under conditions of wound, 100% of individuals registered an accumulation of total phenols content. This accumulation was more significant in hybrids F7926 (171%), F7907 (123%), F7902 (122%) and F7928 (101%). Nonetheless, total phenol content is higher in (I) conditions than in (S) conditions (Figure 1b).

Heterosis effects

Heterosis of hybrid of the F13 and F79 families were estimated, with respect to the average parent; in the hybrid genotypes of F13 family, the manifestation of hybrid vigour was obtained in all treatment conditions. 63, 63 and 50% of hybrid genotypes had a positive heterosis effect under healthy, scarified and inoculated conditions (Table 4). The highest hybrids vigours were manifested by F1315 and F1314 genotypes under inoculated conditions with *P. megakarya* mycelium. For hybrids of F79 family, positive heterosis effects were also observed under all treatment conditions. This effect is respectively 25, 25 and 50% under healthy, scarified and inoculated conditions. These results show that the heterosis effect was more important in F13 family than F79 family (Table 5).

Hierarchical classification of the different genotypes

Total phenols content obtained under different treatment conditions permitted us to hierarchically classify the genotypes of different descendants. The various parental and hybrid groupings of genotypes were realized at 95% of homogeneity.

For F13 family

The direct hierarchical classification of individuals of F13 family permits to distinguish three groups when all treatment conditions are considered. The first group was made up of parent SNK13 and hybrids F1326, F1308, F1324 and F1321. The second was made up only of F1315 hybrid meanwhile the third group included parent T79/467 and hybrids F1314, F1313 and F1307 (Figure 2a). Under inoculated conditions with *P. megakarya* mycelium, two groups were distinguished. Group 1 is made up of parent SNK13 and hybrids F1326, F1321, F1324 and F1308 and the second includes parent T79/467 and hybrids F1313, F1315, F1314 and F1307 (Figure 2b).

For F79 family

The direct hierarchical classification of individuals of F79 family considering all treatment conditions permit distinguishing three groups. Group 1 was made up of hybrids F7911 and F7904. The second was made up of hybrids F7915 and F7919 meanwhile the third group was constituted by parents SNK13 and T79/467, and hybrids F7926, F7928, F7907 and F7902 (Figure 3a).

Under (I) condition only, the total phenols content permits distinguishing two groups. The first group was made up of hybrids F7915, F7904, F7919 and F7911 meanwhile the second was constituted by parents SNK13 and T79/467, and hybrids F7928, F7907, F7926 and F7902 (Figure 3b).

DISCUSSION

In an attempt to minimize the pathogens infection which is a major limiting factor in plant production, resistance parameter was conducted through the evaluation of total phenols content in the different cocoa genotypes based on the degree of tolerance of these genotypes. The main goal of the present study was to analyze the heterosis effect of the phenols content in the healthy pods, scarified and inoculated pods with sterilized agar disc and scarified

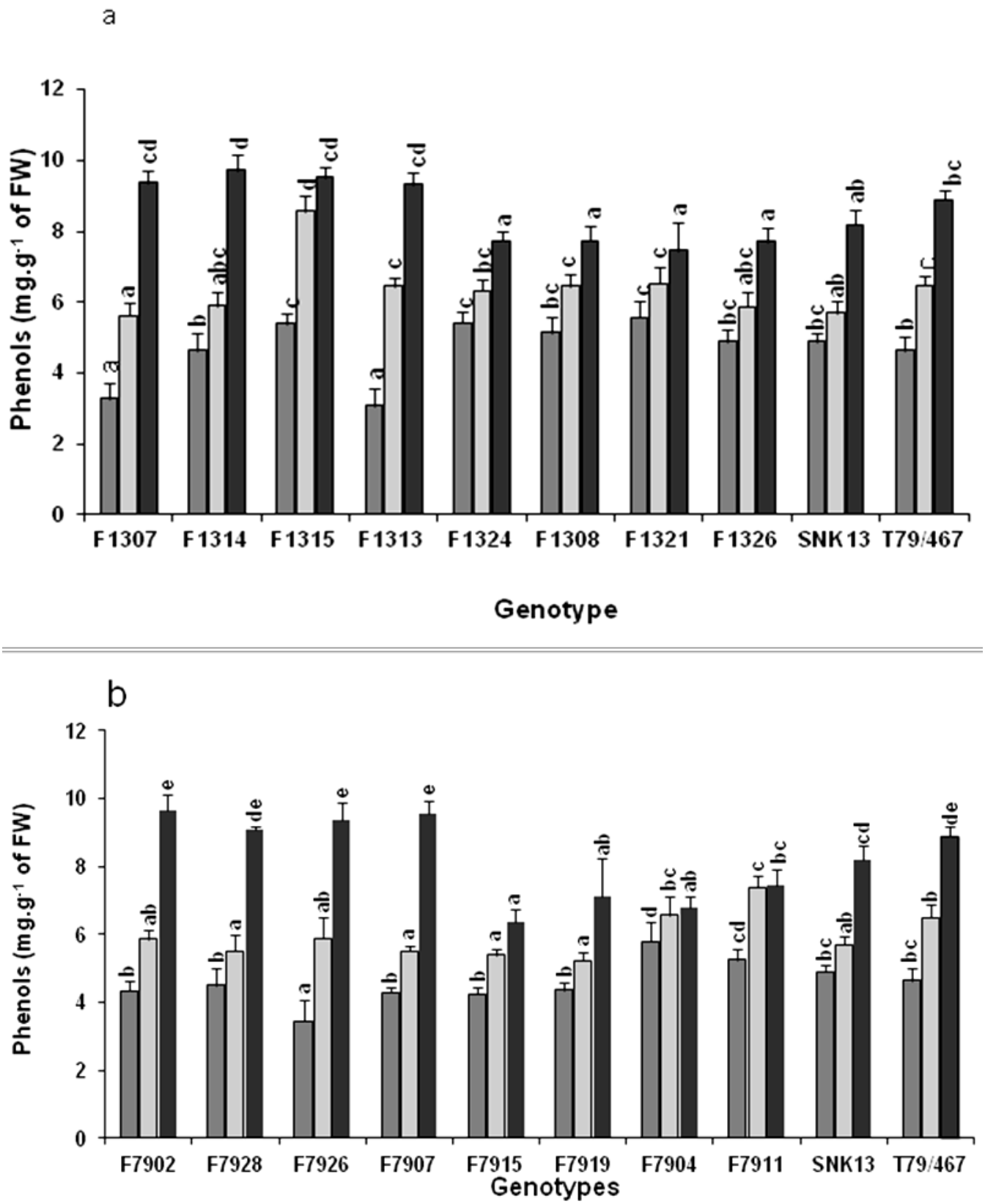

Figure 1. Average content in total soluble phenols in the pods of parental genotypes SNK13 and T79/467 and: (a) hybrids of family F13; (b) hybrids of family F79 under different treatment conditions. Means with the same colors following by the same letter are not significantly different (P< 0.05).

Table 4. Heterosis value (%) with respect to average parents of the phenolic compounds of extracts of F13 family under all conditions.

Genotype	Treatment		
	Healthy	Scarified	Inoculated
F1307	-31.29	-7.74	+9.77
F1314	-2.94	-3.43	+13.82
F1315	+13.29	+40.54	+11.51
F1313	-35.35	+5.97	+9.26
F1324	+13.71	+3.69	-9.46
F1308	+7.75	+5.59	-9.31
F1321	+16.89	+7.07	-12.68
F1326	+2.50	-3.73	-9.65

Table 5. Heterosis value (%) with respect to average parents of the phenolic compounds of extracts of F79 family under different treatment conditions.

Genotype	Treatment		
	Healthy	Scarified	Inoculated
F7902	-9.19	-3.83	+12.82
F7928	-5.42	-9.49	+6.46
F7926	-27.64	-3.41	+9.56
F7907	-10.11	-9.76	+12.12
F7915	-11.11	-11.64	-25.33
F7919	-8.41	-14.27	-16.61
F7904	+21.68	+7.72	-20.56
F7911	+10.21	+21.28	-12.75

and inoculated pods with *P. megakarya* mycelium of parental clones SNK13 and T79/467 and hybrids resulting from the reciprocal crossing of ♀SNK13 x ♂T79/467 in function of the productivity and the resistance of cocoa pod vis-à-vis to *P. megakarya*.

In the point of view of constitution, the pool of phenolic compounds in the tissues of *T. cacao* is a function of genotype. This observation suggests that these metabolic formed part of defense mechanisms. The implication of these phenolic compounds in the mechanism of resistance to pathogenic microorganisms of plants has been demonstrated by Temgo and Boyomo (2002). In this study, the increase of phenolic content under conditions of inoculation with *P. megakarya* mycelium was more abundant in the tolerant parent T79/467 and in 100% of hybrid genotypes. Similar rresults have already been reported by many authors on cocoa leaves inoculated by *P. megakarya* (Boudjeko et al., 2007). These results are also in conformity with those obtained by Baker et al. (2005) who observed an accumulation of phenolic compounds in the extracellular environment of vegetable cells suspensions few hours after interactions between these cells and the pathogenic agent.

Sensitive genotypes accumulate less phenolic compounds under conditions of inoculation compared to tolerant genotypes indicated that this increase is correlated to the degree of genotypes studied. These results are different from those of Boudjeko (2003) who instead found out that the increase in phenolic compounds in the roots of *Xanthosoma sagittifolium* after inoculation by *Pythium myriotylum* seems not to be correlated to the degree of resistance of this plant. In fact, the level of accumulation of phenol in response to inoculation can be correlated to the photosystem and the level of resistance of the host (Nicholson et al., 1992). According to Matern and Kneusal, (1988), the first stage of the defence mechanism of plants against parasitic attacks implies a rapid accumulation of phenolic compounds at the inoculation site. This stops or slows down the progression of the pathogenic agent. During this defence, phenolic compounds can play the role of phytoalexines. They can also contribute to the formation of lignine and contribute as such to the construction of structural barriers to stop the progression of the pathogenic agent. These phenols also have an antioxidant role to counteract prooxidant agents produced during stress caused by pathogenes or the environment (Dixon et Palva, 1995). The highest increases in phenolic compounds were observed in tolerant parent T79/467 and hybrid genotypes F1307, F1314, F1315, F1313,

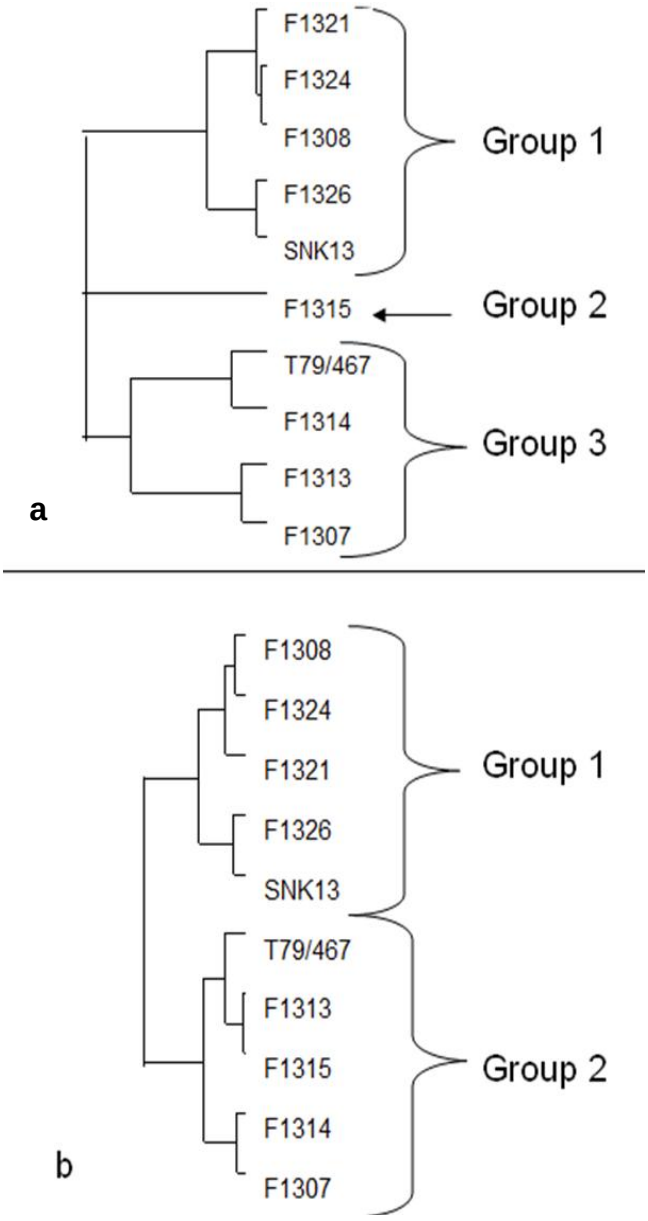

Figure 2. Direct hierarchical classification of the genotypes of F13 family using phenol content under all conditions (a) and under inoculated with *P. megakarya* mycelium alone (b).

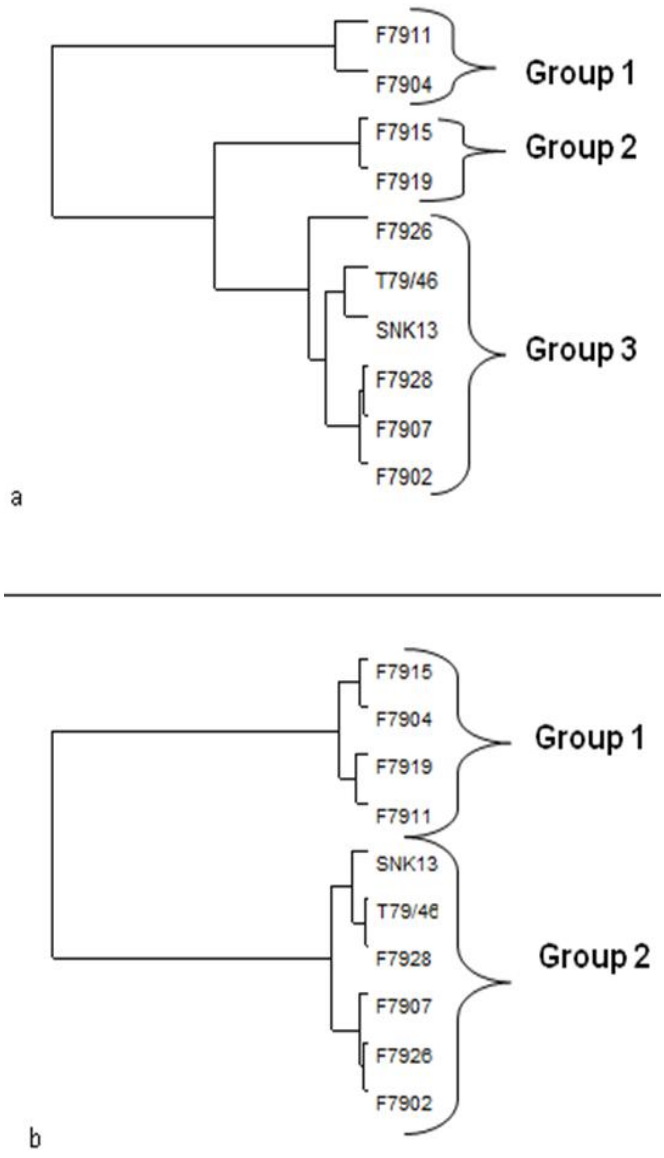

Figure 3. Direct hierarchical classification of the genotypes of F79 family using phenol content under all conditions (a) and under inoculated with *P. megakarya* mycelium alone (b).

F7902, F7928, F7926 and F7907. These genotypes also have the weakest necrosis surfaces. This result is closed to that obtained by Junqueira et al. (2004) who showed an accumulation of fatty acids and phenolic compounds during inoculation in hybrid genotypes of maize. Hierarchical classification of the different genotypes with respect to phenols content enabled 3 and 4 groups for F13 and F79 families at 95% homogeneity. This classification illustrates that about 50% of the progenies manifest hybrid vigour for this trait. This reveals the existence within the progenies of more efficient genotypes than the best parent and that this can be used as parents in future improvement programs (Lockwood et al., 2007). The heteosis effect of each F13 and F79 family when comparing the phenols content revealed a higher variability within both families. In fact, 50% of F13 hybrids and 50% of F79 hybrids presented a posisitive heterosis 6 days after inoculation. Hybrids that present a positive heterosis for a character might have genes containing additive effects in some situations that have an important implication in the transmission of that character.

Conclusion

The evaluation of heterosis effect of phenols in the pods of *Theobroma cacao* allowed the different hybrid genotypes of F13 and F79 families to be compared and classified. This physiological parameter has been analyzed in healthy pods, scarified and inoculated pods with sterilized agar disc (S) and in scarified and inoculated pods with an agar disk containing *P. megakarya* mycelium (I). The results showed that productive and tolerant genotypes (F1307, 1314, F7902 and F7928) have a high phenols content and positive heterosis effect after (I) meanwhile the less tolerant and productive genotypes (F1321, F1326, F7904 and F7911) have a weak content and negative heterosis effect after (I).

Conflict of Interests

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

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Physiological response, molecular analysis and water use efficiency of maize (*Zea mays* L.) hybrids grown under various irrigation regimes

Chigign Adamu[1], Aravinda Kumar B. N.[2*], Rajkumara S.[2], Patil B. R.[3], Patil H. Y.[4] and Kuligod V. B.[5]

[1]Aksum University, Shire Campus, P. O. Box. 314, Shire, Ethiopia.
[2]Department of Agronomy, University of Agricultural Sciences, Dharwad-580 005, India.
[3]Department of Genetics and Plant Breeding, University of Agricultural Sciences, Dharwad-580 005, India.
[4]Department of Crop Physiology, University of Agricultural Sciences, Dharwad-580 005, India.
[5]Department of Soil Science, University of Agricultural Sciences, Dharwad-580 005, India.

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With a view to study the effects of irrigation scheduling on the water use efficiency and physiological response and molecular basis of maize hybrids of different maturity groups, a field experiment was conducted at Water Management Research Center (WMRC), Belvatagi, University of Agricultural Sciences, Dharwad, India during 2010-2011 *rabi* season in Malaprabha Command Area'. The experiment was laid out in split plot design with three replications. The main plot comprised four irrigation levels (0.4. 0.6 and 0.8 irrigation water/cumulative pan evaporation (IW/CPE) ratio and irrigation at critical growth stages of maize) and subplots were three maize hybrids [PEEHM-5 (extra early), PEHM-2 (early) and 900 M gold (full season)] were tested. The results reveal that significantly higher grain yield (P<0.05) was at 0.8 IW/CPE ratio followed by irrigation at critical growth sages of maize. Among the maize genotypes tested, full season 900 M Gold recorded significantly greater grain yield (84.61 q ha^{-1}) over PEHM-2 (early) and PEEHM-5 (extra-early). The moisture stress due to 0.4 IW/CPE ratio prolonged the days which reached 50% anthesis and 50% silking thus widening the anthesis-silking interval. Significant positive correlations (P<0.05) of maize grain yield with 100 seed weight (r=0.81), cob length (r=0.83), harvest index (r=0.82) and water-use efficiency (WUE; r=0.61) were found. The RWC decreased significantly (P<0.05) from 82.53 to 75.24% with increasing moisture stress on account of variations in the availability of soil moisture in the crop root zone. WUE was significantly low (P<0.05) in 0.8 IW/CPE ratio, despite providing more amount of water which could be attributed to a greater use of water with relatively lesser increase in yield. The present investigation shows that providing four irrigations at critical growth stages of maize hybrids followed by either three or five irrigations seem to have higher WUE. This approach could save water up to 29% with slight reduction of grain yield by 12% over providing full irrigation. Molecular analysis of three hybrids revealed the possibility of introgressing the yield enhancing traits from full season hybrid into early and extra-early hybrids, the latter clustered distinctly with each other. This strategy besides saving water helps tail-end farmers in choosing additional crop for double cropping in the command areas.

Key words: Maize hybrids, IW/CPE ratio, water use efficiency, grain yield.

INTRODUCTION

Maize (*Zea mays* L.) a miracle crop, is grown over a wide range of climatic conditions in semi arid and sub-tropics of Indian continent. Besides, it is a water demanding crop; higher grain yields can be achieved when water and nutrients are not limiting. Occurrence of drought is unpredictable as it can occur at any stage of the crop. However, maize is very sensitive to water and other environmental stresses in the period one week before flowering to two weeks after flowering (Grant et al., 1989; Pandey et al., 2000; Cakir, 2004). Drought during this period result in easily measured increase in the anthesis-silking interval (ASI) as the silk emergence is delayed (Zaidi et al., 2007). Further, the water stress occurring at different crop developmental stages could potentially limit biomass accumulation and consequently reduce grain yield of the maize crop.

Throughout the tropics, periodic drought caused by uncertain and ill distributed rainfall and soils with low water holding capacities cause sizeable reduction in maize yield. In India, majority of maize is grown under irrigated conditions and most farmers in south India cultivate maize under rainfed condition also. Significant yield losses in maize from drought are expected to increase with global climate change as temperature rise and rainfall distribution changes in key traditional areas. There is a need to identify suitable management techniques in maize which can withstand water stress situations. Most of the maize grown in the irrigated areas of the Navalgund and Nargund taluks of Dharwad district, Karnataka, India suffers from such water shortages at key developmental stages.

The hypothesis of the study was that under water limited conditions, an early maturing maize hybrid would be a better alternative crop in the area of study. In this context, a field experiment was performed to compare response of maize hybrids of different maturity to varying irrigation schedules in the same location and under the same crop management. Crop development, soil water extraction pattern, biomass and grain yield; and molecular diversity were characterized for maize hybrids. The objectives of this study were: i) to compare agronomic and physiological responses of maize hybrids of different maturity groups to irrigation scheduling; and characterize their molecular diversity and (ii) to quantify the relative yield contribution of maize hybrids and the variations in their water use efficiency (WUE).

MATERIALS AND METHODS

Site description

The field experiment was conducted at Water Management Research Center (WMRC), Belvatagi, University of Agricultural Sciences, Dharwad in Malaprabha Command Area, Karnataka, India during winter season 2010. The experimental site is located in the northern agroclimatic zone (zone-3) of Karnataka at latitude of 15°16' N, and longitude of 75°23' E with an altitude of 579 m above sea level. The soil of the experimental site was analyzed for its physico-chemical properties (Table 1).

The meteorological data gathered during the experimental period are presented in Figure 1. The experimental crop received a very less amount of rainfall (101 mm) during the growing period, only in the month of November. Mean maximum temperature ranged from 31.70 (November) to 37.5°C (March) while the mean minimum temperature ranged from 11.1 (January) to 21.87°C (November). There was an uneven seasonal rainfall distribution coupled with 20.81 mm mean growing season evaporation. The percent relative humidity also declined from November (63.18%) to March (48.96%). Thus, due to frequent drying of top soil (six inches), irrigations were provided based on irrigation water/cumulative pan evaporation (IW/CPE) ratio. The higher temperatures during March resulted in higher evaporation of 6.26 mm which exceeded previous three years average by 0.76 mm (data not shown).

Experimental design and treatments

The experiment was laid out in a split plot design with three replicates using a net plot size of 3.0 x 5.6 m for biometric observations. The maize plants were accommodated in 0.6 m inter-row spacing with 0.2 m intra-row spacing between the plants. Irrigation schedules and maize hybrids were randomized in main and sub-plots, respectively. The treatment combinations comprised four irrigation schedules [I_1 = 0.4 IW/CPE, I_2 = 0.6 IW/CPE, I_3 = 0.8 IW/CPE, and I_4 = irrigations at critical growth stages of maize that is (i) at knee-high stage (V_5 or 35 DAE), (ii) anthesis stage (VT or 65 DAE) and (iii) grain development (R_4 or 90 DAE)]; and three maize hybrids [H_1=PEEHM5 (extra early), H_2 = PEHM2 (early) and H_3 = 900 M Gold (full season)].

Characteristics of maize hybrids used in the study

PEEHM5 and PEHM2 (extra-early and extra maturing hybrids) were released from IARI, India and are recommended for cultivation in Karnataka state. 900 M gold is a full season single cross hybrid of Monsonto Ltd.

Crop husbandry

Maize hybrids were sown on 2nd November 2010 by marking and opening of shallow furrows at 0.6 m apart and seeds were dibbled uniformly at 0.2 m interval in furrows using a seed rate of 25 kg ha^{-1}. Nitrogen, phosphorus and potash were applied at 150, 75 and 37.5 kg ha^{-1}, respectively to all the plots. Entire doses of P_2O_5 and K_2O were applied at planting, while N was applied in three splits that is ⅓ each at the time of sowing, at vegetative stage and before flower initiation stage. The experimental plot was maintained weed free throughout the growth period using pre-emergence

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

Abbreviations: WUE, Water-use efficiency; **RWC**, relative water content; **SPAD,** soil plant analysis development system; **IW/CPE**, irrigation water/cumulative pan evaporation ratio; **DAE**, days after emergence; **RAPD**, random amplified polymorphic DNA.

Table 1. Soil physico-chemical properties.

Soil layer (cm)	Coarse sand (%)	Fine sand (%)	Silt (%)	Clay (%)	Bulk density (g cc^{-1})	Field capacity (%)	Wilting point (%)	Soil pH (1:1.25)	EC (dS m^{-1} at 25°C)	Available N (Kg ha^{-1})	Available P_2O_5 (Kg ha^{-1})	Extractable K_2O (Kg ha^{-1})
0-15	13.20	11.30	14.60	60.90	1.36	40.30	20.60	8.50	0.37	210	35	745
15-30	10.40	13.70	15.70	60.20	1.38	41.25	21.10	8.70	0.30	240	38	741
30-45	10.20	12.80	16.70	60.50	1.39	42.50	21.90	8.80	0.25	235	32	743

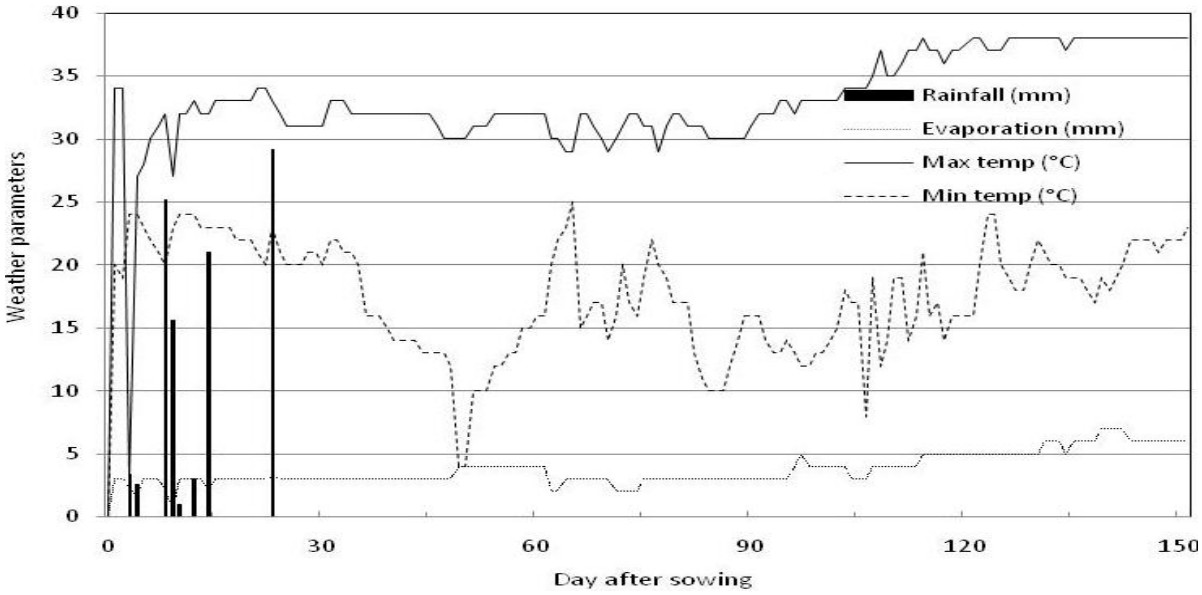


Figure 1. Daily values of rainfall (mm), evaporation (mm), maximum and minimum temperatures (°C) for the period between November, 2010 to March, 2011 (cropping season) at the experimental site.

application of pendimethalin 30 EC at 1.0 kg a.i ha^{-1} followed by manual weeding. Irrigation was applied manually to a depth of 60 mm. The scheduling of irrigation was done based on progressive total of evaporation, after attaining the pre- determined values of cumuli-tive pan evaporation (CPE) (Prihar et al., 1974). Thus, CPE values for different IW:CPE ratios viz., 0.4,0.6 and 0.8 at a constant depth of 60 mm irrigation water (IW) were calculated to be 150, 100 and 75 mm, respectively. The total water use, depth of irrigation water and the number of irrigations provided are presented in Table 2.

Gas exchange measurements and soil plant analysis development system (SPAD) chlorophyll meter values

Leaf gas exchange parameters photosynthesis (P_n), stomatal conductance (g_s), and internal CO_2 concentration (C_i) were measured in the top fully expanded leaf at anthesis stage using a portable infra-red gas analysis system (LI-6400 LICOR, Nebraska, Lincoln, USA) under uniform light conditions. The readings were taken after at an ambient CO_2 concentration of 380 ppm. Three measurements per leaf were taken for each genotype x irrigation combination in each replicate; a total of 48 readings were taken at each time. Gas exchange measurement was taken on a day with sufficient sunlight

Table 2. Total water used (TWU), depth of irrigation water (DIW) and WUE under different irrigation levels.

Treatment	TWU (mm)	DIW (mm)	Number of irrigations
I_1 (0.4 IW/CPE)	240	180	3
I_2 (0.6 IW/CPE)	360	300	5
I_3 (0.8 IW/CPE)	420	360	6
I_4 (critical stages)	300	240	4

and no artificial light source was used for illumination.

Chlorophyll content was determined non-destructively using a SPAD-502 meter (Minolta, Japan), on third fully expanded leaf from the top at 60 DAE (V_{12}) and 90 DAE (R_4) by clamping the SPAD sensor over the leaf lamina. In each plant, five readings were recorded from single leaf.

Soil moisture measurements

Soil moisture was measured gravimetrically before and after irrigation at grand growth, anthesis and at physiological maturity in soil layers: 0 to 15, 15 to 30 and 30 to 45 cm. Soil samples were taken from each plot at about 15 cm away from the crop line. The soil moisture measurements were used to calculate consumptive use and moisture extraction pattern.

Molecular analysis and genomic DNA extraction

Genomic DNA was extracted by cetyltrimethyl ammonium bromide (CTAB) extraction procedure (Doyle and Doyle, 1987). Fresh leaf samples of 1 g were ground to powder in liquid nitrogen and transferred to a 1.5 ml centrifuge tube to which 1 ml of pre-heated (60°C) extraction buffer was added. The extraction buffer consisted of 2% CTAB (w/v), NaCl (4 M), Tris HCl (pH 8.0 1 M) and PVP (0.1%), mercapto ethanol 1% (v/v), RNAse A (2 mg/ml), chloroform: iso-amyl alcohol (24:1) (v/v), ethanol (70%) and TE buffer (Tris HCl, 10 mM (pH 8.0), and 1 mM EDTA (pH 8.0) were the additional solutions required. The samples containing tubes were incubated at 65°C in circulating water bath for 15-20 min. An equal volume of chloroform: iso-amyl alcohol (24:1) was added and mixed for about 5 min. Samples were centrifuged at 12000 rpm for 15 min and the supernatant was decanted and transferred to a fresh tube.

Random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) amplification

Amplifications were carried out using a DNA thermal cycler (Mastercycler gradient, Eppendorf). Each 20 µl reaction volume contained about 50 ng of template DNA, 10X PCR Buffer (Tris with 15 mM $MgCl_2$) [Bangalore Genei, India], 2.5 mM dNTP Mix (Bangalore Genei, India), 10 pmols of single decamer primer (Sigma Genosys, India and Quiazen Operon Technologies, Almeda, USA), 3U/µl of *Taq* DNA polymerase (Bangalore Genei, India).

The PCR programme included an initial denaturation step at 95°C for 5 min followed by 39 cycles with 94°C for 1 min for DNA denaturation, annealing at 31.6°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 8 min were carried out. The amplified DNA fragments were electrophoretically separated on 1.4% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer (for each liters of stock contains 4.84 g of Tris base, 1.14 ml of glacial acetic acid and 2 ml of 0.5 M EDTA) and stained with ethidium bromide (10 mg/ml). Thirty five 10-mer primers randomly selected were used in RAPD analysis. A 250 bp DNA ladder (Bangalore Genie) was used as a marker with molecular size of 5000, 4500, 4000, 3500, 3250, 3000, 2750, 2500, 2250, 2000, 1750, 1500, 1250, 1000, 750, 500 and 250 bp. 20 µl of sample was loaded onto each well and amplified DNA was separated with 70 V constant current for 3 h. The amplified pattern was visualized on a UV trans illuminator and photographed.

Data collection and analysis

Observations on number of days to 50% anthesis, days to 50% silking, anthesis -silking interval, cob length, number of grains per row, above ground biomass, 1000-seed weight, grain yield, harvest index and soil moisture extraction pattern were recorded. Above ground biomass was determined using five plants per plot and the samples were oven dried to a constant weight at 80°C. Antheis silking interval (ASI) was computed as the difference between silking and anthesis dates (Kuchanur et al., 2013). Relative water content (RWC) was measured to determine the plant water status of leaf discs sampled from the third leaf from the top adopting the procedure given by Barrs and Weatherly (1962) as:

RWC (%) = (Fresh weight- oven dry weight) / (Turgid weight-oven dry weight) x 100

The water-use efficiency (kg ha^{-1} mm^{-1}) was estimated in terms of grain yield as the ratio between grain yield (kg ha^{-1}) and total consumptive use of water (mm).

The data collected were analysed using analysis of variance (ANOVA) and Fisher's LSD test to determine the significant differences at $P<0.05$ levels between treatment means. All statistical analyses, except for the molecular analyses were performed with MSTATc (Russel, 1986).

RAPD data analysis and scoring

For RAPD data analysis, the bands with same molecular weight and mobility were treated as identical fragments. RAPD products were scored for presence or absence of each amplicon evaluated. Only those bands that could be unequally scored across all the samples were included in the analysis. Pair wise similarity matrices were generated using Jaccard's coefficient of similarity. Data matrices were prepared in which the presence of a band was coded as 1, whereas the absence as 0. The data matrices were analyzed by the SIMQUAL program of NTSYSpc© (version 2.02j) (Rolf, 1998).

Dendrogram of the similarity coefficients was performed using unweighted pair group method of arithmetic means (UPGMA) through the programme, Popgene Version 1.31 (Microsoft windows based Freeware for population genetic analysis).

RESULTS

Physiological responses of maize hybrids to irrigation scheduling

The comparisons of means of irrigation levels, maize hybrids and their interactions are shown in Tables 3 and 4. The above ground biomass (AGB) at harvest was significantly highest in I_3 (259.56 g plant^{-1}) compared to I_1 (164.96 g plant^{-1}). Averaging across irrigation levels, H_3 produced significantly higher AGB (247.64; P<0.05) over other hybrids. Among interaction effects, H_3 produced more AGB in I_3 compared to other treatments (Table 3). Effect of different irrigation regimes and maize hybrids as well as their combined effect on days to reach 50% anthesis was significant (Table 3). Water stressed regimes I_1 and I_2 resulted in more number of days to reach 50% anthesis. Among hybrids, H_3 took 66.75 days to reach 50% anthesis (P<0.05) over other hybrids. The combined effect also showed a similar trend. A same trend was found for number of days to reach 50% silking. Averaging across all hybrids, the interval between anthesis and silking (ASI) was significantly influenced by irrigation schedules. I_1 extended the ASI (8.4 days) while the least was recorded in I_3 (4.11 days; P<0.05). The effects of hybrids and their combined effects was not significant (Table 3). The cob length, number of grains, 100 seed weight and HI were significantly more in I_1 (14.97 cm, 22.40 g, 28.2 g and 46.22%, respectively) (P<0.05) compared to other irrigation schedules. A similar trend was noticed for these yield components in H_3 (14.44 cm, 34.14 g, 27.5 g and 51.71%, respectively) (P<0.05; Table 3).

Grain yield

Averaging across all the maize hybrids, the yield of I_3 was significantly higher by about 3019 kg ha^{-1} than that of I_1 (Table 3). The yield differences were significant (P<0.05) at all the irrigation levels. The hybrid 900 M Gold (H_3) produced higher grain yields in the range 2143 to 2733 kg ha^{-1} than other hybrids. Among interaction effects, H_3 produced higher grain yields in all the irrigation levels and yield increase ranged from 1077 to 2774 kg ha^{-1}, while for H_1, it was in the range of 810 to 3423 kg ha^{-1}; and for H_2 in the range of 446 to 2860 kg ha^{-1}.

Gas exchange measurements

Irrigation levels did not show significant differences for P_n, g_s, and C_i (P>0.05). Among hybrids, H_1 had the highest C_i (P<0.05) compared to other hybrids. There were significant interaction effects between irrigation levels and maize hybrids for P_n being significantly highest for H_1 at I_4 (P<0.05) (Table 4).

SPAD chlorophyll meter readings

Averaging across hybrids, I_3 recorded maximum SPAD value both at anthesis and grain filling stages (P<0.05). Among hybrids, H_3 recorded the maximum values (P>0.05). There was a trend in response of hybrids to irrigation levels with respect to SPAD values being higher in H_3 at all the irrigation levels (P>0.05) (Table 4).

Relative water content (RWC)

Significantly higher RWC was recorded at anthesis stage in I_3 which was 8.8% higher than I_1 (P<0.05).

Water use efficiency (WUE)

I_4 had significantly the highest value of 23.80 kg ha^{-1} mm (P<0.05). Providing water at higher frequency, I_3 resulted in decrease in WUE by 4.5 kg ha^{-1} mm. Over all, the WUE for maize hybrids was in the range 17.25-26.16 kg ha^{-1} mm. There were significant interactions between irrigation levels and maize hybrids being highest with H_3 at I_1 (30.12 kg ha^{-1} mm) but was at par with H_3 at I_4 (29.75 kg ha^{-1} mm) (Table 3).

Soil moisture extraction pattern

At sowing, the soil profile was close to field capacity in all the plots. The depletion of soil moisture was higher in the top soil layer due to delayed irrigation (I_1) both at vegetative stage and anthesis stage. On the contrary, lowest depletion was found at all the soil depths in I_3. The interaction effects were not significant (P>0.05) (Data not shown).

RAPD analysis

Random amplified polymorphic DNA analysis of three maize hybrids on 34 primers produced a total of 351 amplified fragments, 202 of which were polymorphic, and the percentage of polymorphism was 57.55. These amplified fragments ranged in size from 250 to 5000 bp (Figure 2). On average, 10.32 bands were amplified per primer and 5.94 were polymorphic (Table 6). Jaccard's coefficient of similarity ranged from 0.70 to 0.74. A highest genetic diversity was observed between H_1 and H_3 (0.73). The dendrogram revealed two distinct clusters; H_1 and H_2 clustered distinctly away from H_3.

DISCUSSION

Effect of irrigation scheduling on physiological responses of maize hybrids

Results of this study show higher grain yield with 0.8 IW/CPE (I_3) on account of higher cob length, cob girth, number of grains per row, and 1000- seed weight. The

Table 3. Yield and yield components of maize (*Zea mays* L) under different irrigation levels.

Treatment	Above ground biomass (g plant^{-1})	Days to 50% anthesis	Days to 50% silking	Anthesis-silking interval	Cob Length (cm)	No. of grains row^{-1}	100 seed weight (g)	Grain yield (kg ha^{-1})	Harvest index (%)	Water use efficiency (Kg/ha-mm)
Irrigation levels										
I_1 : 0.4 IW/CPE	164.96	61.40	69.30	8.44	10.80	20.28	23.33	5124	39.22	21.25
I_2 : 0.6 IW/CPE	230.25	61.30	66.60	5.11	14.07	21.79	25.36	6909	46.10	19.13
I_3 : 0.8 IW/CPE	259.56	60.30	64.60	4.11	14.97	22.40	28.20	8143	46.22	19.29
I_4 : Critical Stage Irrigation	240.27	60.90	66.31	5.22	14.22	21.04	25.23	7168	43.03	23.80
LSD (*P=0.05*)	8.00	0.20	0.32	0.22	0.82	0.29	0.47	1260	0.83	0.42
Maize hybrids										
H_1: PEEHM-5	206.14	57.75	63.92	6.08	12.23	14.55	24.23	5728	36.00	17.25
H_2: PEHM-2	217.50	58.50	63.92	5.58	13.88	15.44	24.80	6318	43.22	19.19
H_3 : 900 M Gold	247.64	66.75	72.25	5.50	14.44	34.14	27.57	8461	51.71	26.16
LSD (*P=0.05*)	2.61	0.18	0.22	ns	0.52	0.19	0.42	720	0.42	0.23
Interaction (I x H)										
I_1 x H_1	148.45	59.00	67.67	9.33	7.90	14.33	22.90	3527	27.85	14.63
I_1 x H_2	158.68	58.00	65.00	8.00	12.07	15.63	23.07	4582	36.98	19.00
I_1 x H_3	187.74	67.33	75.33	8.00	12.43	30.87	24.03	7262	52.84	30.12
I_2 x H_1	196.48	57.33	63.67	5.67	13.10	14.67	23.10	6143	39.46	17.01
I_2 x H_2	226.53	60.00	64.33	4.67	14.33	15.53	24.73	6996	46.39	19.37
I_2 x H_3	267.74	66.67	71.67	5.00	14.77	35.17	28.23	7587	52.46	21.01
I_3 x H_1	247.97	56.67	60.67	4.00	13.60	14.80	27.27	6950	39.43	16.46
I_3 x H_2	243.22	58.00	62.67	4.33	14.80	15.30	25.83	7442	46.85	17.63
I_3 x H_3	287.49	66.33	70.33	4.00	16.50	37.10	31.50	10036	52.39	23.77
I_4 x H_1	231.65	58.00	63.67	5.33	14.30	14.40	23.63	6292	37.26	20.90
I_4 x H_2	241.58	58.00	63.67	5.33	14.30	15.29	25.57	6251	42.68	20.76
I_4 x H_3	247.59	66.67	71.07	5.00	14.07	33.43	26.50	8959	49.16	29.75
LSD (*P=0.05*)	10.42	0.72	0.89	ns	2.06	0.75	ns	2890	1.69	0.92

ns = Not significant

significant increases in these yield components were due to beneficial effect of sufficient moisture available in the soil. This result is in conformity with the findings of Farshad et al. (2008) who showed that missing single irrigation at any of the growth stages in maize significantly decreases grain yield. Scheduling irrigation at critical stages of growth also significantly improved grain yield than providing more irrigation in I_3. The moisture stress encountered in I_1 resulted in more number of days to reach 50% anthesis, days to reach 50% silking and thus widening the interval between anthesis and silking. Continuous stress due to low frequency of irrigation in I_1 also prolonged the days to reach 50% silking by about eight days. In

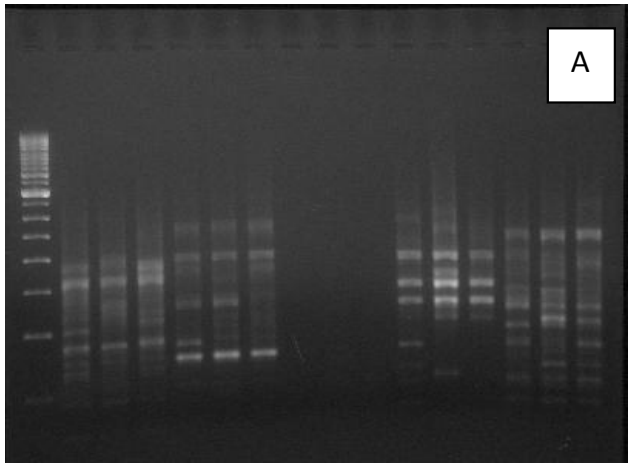
Table 4. Relative water content (RWC), SPAD values and stomatal aperture traits of maize (*Zea mays* L) under different irrigation levels.

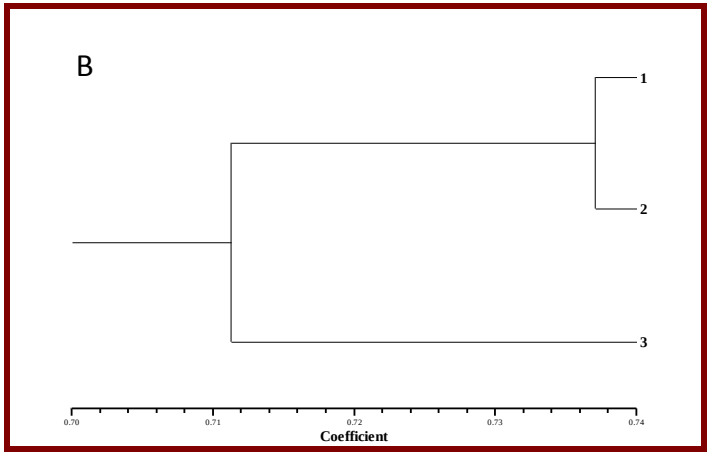
Treatment	RWC at anthesis (%)	SPAD values	Rate of photosynthesis (µ mole m^{-2} s^{-1})	stomatal conductance (m mole cm^{-2} s^{-1})	internal CO_2 concentration (ppm)
Irrigation levels					
I_1 : 0.4 IW/CPE	75.24	29.77	6.48	26.50	148.93
I_2 : 0.6 IW/CPE	76.68	36.87	9.43	28.67	134.50
I_3 : 0.8 IW/CPE	82.53	41.29	7.19	25.17	240.46
I_4 : Critical Stage Irrigation	79.52	39.63	12.69	49.67	155.74
LSD (*P=0.05*)	1.34	1.15	ns	ns	Ns
Maize hybrids					
H_1: PEEHM-5	77.74	35.40	9.08	32.75	198.09
H_2: PEHM-2	78.74	36.10	8.75	35.25	168.01
H_3 : 900 M Gold	79.00	39.17	9.01	29.50	143.62
LSD (*P=0.05*)	ns	0.55	ns	ns	9.23
Interaction (I x H)					
I_1 x H_1	73.44	29.05	5.30	21.50	143.77
I_1 x H_2	74.30	28.20	8.54	37.00	168.51
I_1 x H_3	77.99	32.05	5.60	21.00	134.51
I_2 x H_1	72.71	35.29	8.20	27.50	155.24
I_2 x H_2	79.02	35.93	7.90	29.00	133.40
I_2 x H_3	78.33	39.37	12.21	29.50	114.85
I_3 x H_1	84.14	39.31	6.79	29.00	258.56
I_3 x H_2	80.61	41.20	6.56	26.50	234.61
I_3 x H_3	82.84	43.37	8.22	20.00	228.21
I_4 x H_1	80.67	37.94	16.04	53.00	234.78
I_4 x H_2	81.04	39.07	12.03	48.50	135.54
I_4 x H_3	76.85	41.88	10.02	47.50	96.91
LSD (*P=0.05*)	NS	ns	ns	ns	ns

ns = not significant

maize, a wider ASI causes poor synchronization of flowers leading to decline in grain yield. Kuchanur et al. (2013) reported that in maize, moisture stress increased significantly the days required for 50% anthesis, 50% silking and ASI. In this study, the association of ASI with grain yield was significantly negative (r= -0.73; P<0.05) (Table 5). Monneveux et al. (2006) reported in two drought tolerant populations viz. DTP1 and DTP2 that significant yield gains in the populations were associated with a significant increase in number of cobs per plant and grains per ear and significant reductions in ASI. Further, it is evident in the literature that the shortening of ASI is associated with high grain yield under drought (Edmeades et al., 2000; Moser et al., 2006). The increase in grain yield of H_3 was about 32.30% over H_1 and 26.23% over H_2. This might be due to genetic and morphological characteristics of maize hybrids exploiting climatic maxima at important growth stages. In the present study, all the yield traits have contributed for yield increment with significant positive correlations for 1000-seed weight (r= 0.81); cob length (r= 0.83); harvest index (r=0.82); SPAD values (r=0.79) and WUE (r=0.61) (P<0.05) (Table 5). H_3 exhibited a higher HI which might be due to a genetically strong source-sink relation resulting in higher yields. A higher total biomass production in well watered situations has been reported by Moser et al. (2006). Among interaction effects, H_3 performed equally well in all the irrigation levels. The grain yield ranged from 4582 to 7442 kg ha^{-1} in H_2 and from 3527 to 6950 kg ha^{-1}. In this study, lesser number of irrigations resulted in reduction in grain number and 100 grain weight. This is in agreement with the findings of Moser et al. (2006) which showed a decrease in kernel number and 1000-kernel weight resulted in lower grain yields due to water shortage in maize.

The RWC decreased significantly (P<0.05) with increasing moisture stress. I_3 had higher RWC by about 82.53% compared to I_1 (75.24 %). This may be attributed to better availability of soil moisture in the crop root zone. The earlier findings in maize revealed that, water potential and RWC (Chen et al., 1990) and relative water content (Schlemmer et al., 2005; Kuchanur et al., 2013)





C	*Maize hybrids*	PEEHM5	PEHM2	900 M Gold
PEEHM5		**1.00**		
PEHM2		**0.74**	**1.00**	
900 M Gold		**0.70**	**0.73**	**1.00**

Figure 2. (A) Amplification of RAPD products from three different maize hybrids (PEEHM5; PEHM2; 900 M Gold) with 4 primers OPP 2, OPP 3, OPP 5, OPP 11. Lane M = 250 bp DNA Marker. (**B)** Dendrogram of the three different maize hybrids (1=PEEHM5; 2= PEHM2; 3=900 M Gold) obtained by RAPD using UPGMA method. **(C)** Similarity matrix computed with Jaccard's coefficient of three maize hybrids obtained from RAPD markers.

Table 5. Associations of growth and yield traits of maize (*Zea mays* L) with grain yield under different irrigation levels.

Correlation	Above ground biomass (g plant^{-1})	Days to 50% anthesis	Days to 50% silking	Anthesis silking interval	Cob length (cm)	Number of grains row^{-1}	100 seed weight (g)	Harvest index (%)	SPAD values	WUE Kg/ha-mm
Grain yield (kg ha^{-1})	0.85**	0.64*	0.37^{ns}	-0.73**	0.83**	0.73**	0.81**	0.82**	0.79**	0.61*

**Indicates significance level P<0.01 ns = not significant

declined under low water conditions. The chlorophyll concentration is a measure of functional stay green (Barker et al., 2005). The chlorophyll content as measured by SPAD values decreased under water stress but it was more drastic under I_1. A higher photosynthetic rate was found in I_4 being highest with H_1 (16.04 µ mole m^{-2} s^{-1}). The other parameters of stomatal aperture traits were not significant. Moderate stress did not significantly change the relative water content (RWC). Severe stress at silking stage did significantly decrease the leaf RWC and increase leaf

Table 6. Total number of amplicons, number of polymorphic bands and per cent polymorphism of maize hybrids.

Primer	Sequence (5' → 3')	Total number of bands (a)	Number of polymorphic bands (b)	Percent polymorphism (b/a*100)
OPP-02	TCGGCACGCA	13	11	84.62
OPP-03	CTGATACGCC	10	08	80.00
OPP-05	CCCCGGTAAC	12	09	75.00
OPP-06	GTGGGCTGAC	13	06	46.15
OPP-07	GTCCATGCCA	10	04	40.00
OPP-08	ACATCGCCCA	14	09	64.29
OPP-09	GTGGTCCGCA	16	12	75.00
OPP-10	TCCCGCCTAC	18	11	61.11
OPP-11	AACGCGTCGG	13	06	46.15
OPP-12	AAGGGCGAGT	09	02	22.22
OPP-13	GGAGTGCCTC	08	04	50.00
OPP-14	CCAGCCGAAC	08	03	37.50
OPP-15	GGAAGCCAAC	05	00	0.00
OPP-16	CCAAGCTGCC	07	02	28.57
OPP-17	TGACCCGCCT	10	06	60.00
OPP-19	GGGAAGGACA	04	01	25.00
RKAZ-1	TCGGATCCGT	10	06	60.00
RKAZ-3	GGCTGTGTGG	12	08	66.67
RKAZ-4	GGCTGTGTGG	11	07	63.64
RKAZ-5	GGCTGTGTGG	06	02	33.33
RKAZ-8	TCGCTCGTAGS	05	04	80.00
RKAZ-9	CGCTCGCGCT	08	03	37.50
K-01	CATTCGAGCC	9	06	66.67
K-06	CACCTTTCCC	16	08	50.00
K-07	AGCGAGCAAG	06	01	16.67
K-08	GAACACTGGG	11	06	54.55
K-09	CCCTACCGAC	12	07	58.33
K-10	GTGCAACGTG	07	05	71.43
K-11	AATGCCCCAG	11	07	63.64
K-12	TGGCCCTCAC	11	07	63.64
K-13	GGTTGTACCC	10	08	80.00
K-14	CCCGCTACAC	19	15	78.95
K-17	CCCAGCTGTG	08	02	25.00
K-18	CCTAGTCGAG	09	06	66.67
	TOTAL	351	202	1832.28
	Average	10.32	5.94	57.55

relative conductivity (Li-Ping et al., 2006).

A highest WUE was observed at I_4 (23.80 kg ha^{-1} mm) than I_1 (21.25 kg ha^{-1} mm). The increased water application resulted in increase in crop water use without a corresponding increase of yield which was reported by Kar and Verma (2005). Providing irrigation at critical stages that is I_4 resulted in better grain yield and WUE over I_2 on account of optimum number of irrigations. This is in agreement with the findings of Jiotode et al. (2002) which revealed better WUE with irrigation at critical growth stages of maize. While Maqsood et al. (2012) reported that providing six irrigations at different growth stages of maize along with higher N rates up to 200 kg ha^{-1} has increased maize grain yield. WUE values for rainfed maize have been reported in the literature in the ranges 11.4 to 14.4 kg $ha^{-1}mm^{-1}$ (Meena et al., 2009); 9.3 to 13.8 kg $ha^{-1}mm^{-1}$ (El-Tantawy et al., 2007); and 11.0 to 18.0 kg $ha^{-1}mm^{-1}$ (Tijani et al., 2008).

Despite providing a highest amount of water (420 mm) in I_3, the WUE was significantly low ($P<0.05$) which could be attributed to a greater use of water with relatively lesser increase in yield. Trooijen et al. (1999) found greater WUE of maize with limited irrigation, but full irrigation of maize was more profitable than limited irrigation. During vegetative growth stages, soil moisture depletion from different soil layers varied with irrigation

levels. Moisture extraction was more from the top layer (0-15 cm) irrespective of irrigation levels. However, it was more at I_1 coupled with moisture loss through evaporation from soil. Similar trends were found during anthesis stage except in I_3 wherein depletion of moisture from 15 to 30 cm depth was seen. Maximum moisture extraction from deeper layers may be due to stress as a result of less number of irrigation which encourages more rootgrowth into deeper profiles. It is also reported in other study that drought sensitive inbred maize lines despite having deeper rooting markedly reduced WUE on account of inefficient photosynthesis (Hund et al., 2008).

Based on the water extraction patterns, the lack of significant differences in water extraction at depth among maize hybrids and their interaction with water levels was mainly due to a well developed root system by maize. The relationship between yield and irrigation is affected by factors such as climate, soil properties and irrigation practices (Tolk and Howell, 2003) and determining the level of irrigation needed to optimize profits can be complex and depends on both biophysical and economic factors (English et al., 2002; Payero et al., 2008).

Conclusion

The study focuses to bring interrelations between different maturity group hybrids and their WUE through several physiological, molecular and agronomic analyses. Our findings show that providing four irrigations at critical growth stages followed by either three or five irrigations seem to have higher WUE. This approach could save water up to 29% with slight reduction of grain yield by 12% over providing full irrigation. Further, WUE was increased to a maximum of 23.80 kg ha^{-1} mm with decreased frequency of irrigation at critical growth stages. The RAPD analysis revealed that extra-early and early maturity hybrids clustered significantly differently from full season hybrid. This diversity may be of use in crop improvement to introgress certain traits from full season hybrid into early maturity groups for enhanced productivity while emphasizing on the reduction in number of days to reach physiological maturity. To integrate differential responses during phenological stages, it is suggested that field research be combined with thoroughly calibrated and validated crop-water productivity models to further improve strategies obtained from field experiments. In areas where water supply is going to be a constraint in future, farmers must choose varieties and irrigation strategies to ensure sustainable production. Under these situations per day productivity of crop both based on crop duration and on water use need to be given due consideration with a view to save water resources as well as to have temporal benefit so that farmers will have an option to choose additional crop for inclusion in the double cropping system. This perhaps is the only alternative that net returns can be maximized, especially for the farmers in tail-end regions of canal areas.

Conflict of Interest

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

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Antimicrobial activities of lactic acid bacteria isolated from akamu and kunun-zaki (cereal based non-alcoholic beverages) in Nigeria

Okpara, A. N., Okolo, B. N. and Ugwuanyi, J. O.*

Department of Microbiology, University of Nigeria, Nsukka, Nigeria.

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Three lactic acid bacteria (LAB) isolates designated AS_1, AS_2 and KN_4 isolated from kunun-zaki (a sorghum based non-alcoholic beverage widely consumed in Northern Nigeria) and identified as *Lactobacillus plantarum, Lactobacillus brevis* and *Lactobacillus delbruckii*, respectively, produced significant inhibitory compounds in broth. The partially purified inhibitory compounds were screened by agar spot assay method for antagonistic activity against target Gram positive and negative bacteria as well as yeasts associated with food spoilage. The partially purified compounds exhibited strong activity against *Staphylococcus aureus* ATCC 12600, *Bacillus cereus* and *Escherichia coli* ATCC 11775. The inhibitory compound produced by AS_1 and KN_4 inhibited *Bacillus subtilis.* Only the inhibitory compound produced by AS_1 affected *Candida albicans* and *Candida krusei.* Analysis of variance indicated that there was a significant difference ($P<0.05$) in the susceptibility of the different target organisms to partially purified inhibitory compounds. Gram positive bacteria were affected more than yeasts. Proteolytic enzymes, trypsin and pepsin, but not catalase and α-amylase, completely inactivated antagonistic activity of the compounds demonstrating their proteinacious nature. The inhibitory compounds were fairly heat stable and also stable over broad pH ranges. The use of these or related GRAS isolates in the production of this and related beverage may increase the safety, shelf life and marketing appeal of such beverages

Key words: Bacteriocins, lactic acid bacteria (LAB), target organisms, antimicrobial activity.

INTRODUCTION

Lactic acid bacteria (LAB) play essential roles in the fermentative production of many traditional foods. A wide variety of strains are routinely used as starter cultures in the manufacture of fermented dairy, meat, vegetable and bakery products (Lowe and Arendt, 2004). Many LAB strains are present as natural contaminants on a variety of foods such as cereals (De Martinis et al., 2001; kalolou et al., 2004), vegetables (Garcia-Graellis et al., 2000; Buddle et al., 2003; Ogunbanwo et al., 2004), milk (Kalchayanand et al., 1994; Gould, 1996) and meat (Moreno et al., 1999; Mataragas et al., 2003; Cadirci and Citak, 2005). In some parts of Africa, they play important parts in the production of alcoholic beverages and cereal based weaning formulae. Although, they may represent

*Corresponding author. E-mail: jerry.ugwuanyi@unn.edu.ng. Tel (+234) 0 803 306 6518.

undesirable contaminants in some food products, LAB, through their fermentative activities also exert a positive effect, imparting desirable flavours and inhibiting a variety of food spoilage and pathogenic organisms (Strom et al., 2002; Buddle et al., 2003).

The antimicrobial effect of LAB has been used by man through fermented foods for more than 10,000 years without any adverse effects (Shehane and Sizemore, 2002; Soomro et al., 2002) and this has enabled him to fortuitously improve the shelf life, safety and nutritional status of many foods. The preservative effect of LAB is partly due to production of a number of antimicrobial metabolites including organic acids, hydrogen peroxide and diacetyls (Adams and Moss, 1997; O'Keeffe and Hill, 1999; Vaughan et al., 2004). Among the various antimicrobial metabolites produced by LAB, bacteriocins are often the most potent inhibitors of bacteria (Klaenhammer, 1988; Deegan et al., 2006). Bacteriocins and bacteriocin-producing strains of LAB have been the focus of extensive research in recent years due to their food preserving potential (Toora, 1995; Savadogo et al., 2004). Although bacteriocins are produced by a broad spectrum of bacteria, those produced by LAB are of particular interest in the food industry because these bacteria have generally been recognized as safe (GRAS) (Nettles and Barefoot, 1993). Furthermore, as the majority of bacteriocin-producing LAB is natural food isolates, they are ideally suited to food applications (Deegan et al., 2006).

Although, various methods are employed for the preservation of foods, increasing consumer awareness of the uncertainties and potential health risks associated with the use of synthetic chemicals as preservatives in food has made it necessary to examine the possibility of using antimicrobial agents of biological origin as biopreservatives in food industries. Of particular relevance in this connection is the use of antimicrobial preservatives produced by the GRAS microorganisms employed in the production of the foods in question. This study is therefore, designed to screen Akamu and Kunun-zaki (cereal based non-alcoholic beverages) widely consumed in Nigeria for bacteriocin-producing LAB and to examine the partially purified bacteriocins for their capacity to inhibit the growth of selected spoilage and food-borne pathogens.

MATERIALS AND METHODS

Akamu was purchased from Nsukka market as produced and sold by local producers, while Kunun-zaki was purchased from hawkers in Enugu all in Enugu State of Nigeria. Both products were produced and marketed by peasant domestic producers. Five samples of each beverage were bought.

Target organisms

Spoilage and food-borne microorganisms (target organisms) used in this study were *Staphylococcus aureus*, ATCC 12600, *Escherichia coli*, ATCC 11775 obtained from Bioresources Development and Conservation Programme (BDCP) Centre, Nsukka. Untyped strains of *S. aureus* (4 strains), *Bacillus subtilis, Candida albicans,* and *Candida krusei* were obtained from the culture collection of the Department of Microbiology, University of Nigeria, Nsukka. Pathogenic organisms were grown aerobically in tryptic soy broth supplemented with 0.6% (w/v) yeast extract (TSB/YE) at 37°C. Food spoilage organisms were grown in nutrient broth under the same condition. The organisms were maintained by weekly subculturing on slants of appropriate media and stored at 4°C. Before each experiment, the microorganisms were activated by successive subculturing, and 18 to 24 h culture of each target organism was used for bioassay. Culture of target organism used in bioassay was standardized at approximately $5x10^7$cfu/ml determined by standard pour plate method on appropriate media.

Isolation of LAB from 'Akamu and kunun-zaki'

Sample homogenates of akamu and kunun-zaki were prepared and inoculated on de Mann, Rogosa and Sharp, (MRS) agar (Fluka) as described by Oxiod (1982). Inoculated plates were incubated at 37°C for a maximum of 72 h in a candle jar and the developed colonies counted. LAB colonies were purified by repeated subculturing on MRS agar. Pure cultures of LAB isolates were stored as frozen culture in MRS broth supplemented with 25% sterile glycerol, while working cultures were maintained on MRS agar slants at 4°C.

Screening and selection of LAB isolates with antagonistic activity

Pure cultures of LAB isolates were screened for antagonistic activity against target organisms by agar spot method. Overnight culture of each isolate of LAB was spotted onto MRS agar plate and incubated at 37°C for 24 h in a candle jar to allow colonies to develop. Seven milliliters of semi-solid TSB/YE (containing 0.7% agar) was incubated with approximately 5×10^7 cells/ml of target organism to be tested for sensitivity to LAB culture and overlaid on MRS agar plates on which LAB isolates were grown in triplicate. Another set of triplicate MRS agar plates containing LAB colonies were overlaid with 7 ml of sterile TSB/YE (0.7% agar) as control. Plates were incubated aerobically at 37°C for 24 h and examined for zones of inhibition. The colonies that showed inhibition zones were selected and characterized.

Characterization and identification of LAB Isolates

Pure cultures of selected LAB isolates were characterized as described by Batt (1999) and Teixeira (1999). The following standard microbiological tests were used for characterization of isolates, microscopic examination of cell morphology, physiological tests, biochemical tests, cultural growth conditions and carbohydrates (sugar) fermentation profile. Identification was based on comparison of observed characteristics of isolates with those of lactic bacteria as described in the Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Production and assay of bacteriocin

For bacteriocin production, 1000 ml of MRS broth (without Tween 80) in 1.2L Erlenmeyer flasks were inoculated with 1% (v/v) of overnight culture of each LAB isolate and incubated without agitation at 37°C for 48 h in a candle jar. After incubation, cells were removed by centrifugation at 6000 *g* for 10 min at 4°C, followed

by filtration through 0.45 mm pore size cellulose acetate filter to obtain a cell-free supernatant (CFS). The crude bacteriocin present in the culture supernatant was precipitated by ammonium sulphate (40% saturation) at 4°C. The precipitate (crude bacteriocin) was partially purified by dialysis against 20 M sodium phosphate buffer (pH 7.0) at 4°C for 2 h using standard protein dialysis bags (BDH, Poole England). The partially purified bacteriocin was sterilized by passing through a 0.45 µm membrane filters and stored at 4°C for further use without any other treatment unless otherwise stated.

Determination of antimicrobial activity of bacteriocin

The inhibitory activities of bacteriocins against target microorganisms were determined by agar well diffusion method (Choi et al., 1999). Antimicrobial assay was performed under conditions that eliminated the inhibitory effects due to other compounds such as organic acids and hydrogen peroxide (also produced by LAB). Dialysis eliminated organic acids while the effects of hydrogen peroxide was excluded by addition of catalase (sigma LGI 026k 7049) in the concentration of 1mg per ml. To exclude the inhibition due to the presence of lytic bacteriophages, the reverse side technique was used according to Moreno et al. (1999). For bacteriocin assay, 7 ml of soft TSB/YE (0.7% agar) was inoculated with 100 µl of overnight culture containing 5 ×10 cells/ml of target organism and overlaid on TSB/YE (1.5% agar) plates in duplicate and allowed to gel.

Two wells of 5 mm diameter were cut in the agar plate using a sterile cork borer. Wells were filled with 100 µl of bacteriocin solution. Another set of duplicate TSB/YE agar plates inoculated in the same way except that their wells were filled with 20 M sterile sodium phosphate buffer (pH 7.0) in place of bacteriocin were prepared as control. After incubation, plates were examined for the presence of inhibition zones around the wells as an indicator of inhibitory activity. The diameters of the inhibition zones were measured in millimeters. Antagonistic activities were expressed and recorded as the mean of inhibition zone values.

Determination of total soluble protein in the bacteriocin solution

Total soluble protein in the bacteriocin solution was estimated by the method of Lowry (1951), using bovine serum albumin (BSA) as standard. A reagent blank containing 20 M sodium phosphate buffer in place of bacteriocin was also prepared. Colour development was measured at 595 nm in spectrophotometer (LKB Biochem Novaspec, Cambridge England), immediately after incubation.

Effects of hydrodytic enzymes on stability of bacteriocin

The nature of the inhibitory compound produced by LAB isolates was investigated by testing their sensitivity to some hydrolytic enzymes. The enzymes used were Trypsin from beef pancreas (EC No: 232-6508, Breckland Scientific, U.K), pepsin (EC: 232-629-3, Breckland Scientific, U.K), catalase from bovine liver (Sigma, I G, 026k 7049) and α-amylase (EC. No: 336-5656, Breckland Scientific, U.K).

Each inhibitory compound was treated with the various enzymes as follows: each enzyme was suspended in appropriate buffer solution at concentration of 5 mg/ml. Trypsin was suspended in 0.1 M tris hydrochloride buffer (pH 7.2), pepsin in 0.1 M citrate buffer (pH 6.2), catalase in 20 M sodium phosphate buffer (pH 7.0) and α-amylase in 0.1M citrate phosphate buffer (pH 6.2). Afterwards, each sample of bacteriocin was treated with the different enzymes solution to a final concentration of 1 mg/ml. Bacteriocin-enzyme mixture was incubated for 1 h at room temperature (≈28°C). Following enzyme treatment, the antagonistic activity of each inhibitory compound against a sensitive target organism, *S. aureus* ATCC 12600 was monitored by agar well diffusion assay as previously described. Untreated bacteriocin samples were used as control.

Effects of temperature on stability of bacteriocin

Each sample of bacteriocin was tested for stability at different temperatures. Aliquot of each sample freshly obtained from LAB isolates was heated at different temperatures ranging from 40 to 80°C for 30 min, with increment of 10°C and also at 100°C for 15 min. Following heat treatment, samples were cooled to room temperature. Afterwards, the residual activity of the heat treated samples against *S. aureus* ATCC 12600 was monitored by agar well diffusion assay as previously described and compared to the activity of the untreated samples used as control.

Effects of pH on stability of bacteriocin

Each partially purified bacteriocin sample was screened for stability at different pH values (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10, 0). The initial pH of sample (pH 7.0) was adjusted to pH 2, 3, 4, 5 and 6 with 0.1 M hydrochloric acid (HCL) and pH 8, 9 and 10 with 1 M sodium hydroxide (NaOH) and subsequently incubated at room temperature (≈28°C) for 1 h. Thereafter, the residual activity of treated sample against *S. aureus* ATCC 12600 was monitored by agar well diffusion method as previously described. The activity of sample at initial pH (7.0) was used as a control.

Statistical analysis of data

The antagonistic activities of LAB isolates and partially purified bacteriocin from LAB isolates as well as the effects of various physicochemical parameters on the stability of bacteriocin were evaluated using the two-way analysis of variance (ANOVA) and student's t-test. Duncan's multiple range tests of variables was used for comparisons between antagonistic activity and susceptibility of a target organism. It was also used to identify means that differed significantly. The differences were considered significant at $p \leq 0.05$.

RESULTS

Prevalence of LAB in samples of akamu and kununzaki

Data obtained from this study indicate that LAB occurred in high numbers on cereals. The viable cell colony count on MRS agar ranged from 10^7 to 10^{10} colony forming unit per gram or millilitre (cfu/g or cfu/ml) of sample. A total of 13 presumed LAB isolates were obtained from the beverages sampled. Out of these, eight isolates were from akamu and five from kunun-zaki. Of the 13 isolates, three (designated AS_1, AS_2 and KN_4) were selected for further studies on the basis of appreciable antagonistic activity against target organisms on preliminary assays. Isolates AS_1 and AS_2 were obtained from Akamu while isolate KN_4 was from Kunun-zaki.

Table 1. Morphological, physiological and biochemical characteristics of isolates.

Test	AS_1	AS_2	KN_4
Gram reaction	+	+	+
Microscopic appearance	Bacilli	Short rods	Bacilli
Cellular arrangement	Chains	Pairs	Chains
Motility	-	-	-
Spore	-	-	-
Catalase	-	-	-
Hydrolysis of arginine	-	+	+
Growth in 18% NaCl	-	-	+
Growth in 6.5% NaCl	+	+	+
Growth at pH 9.6	-	-	-
Growth at pH 4.4	+	+	+
Growth at 10°C	-	-	-
Growth at 45°C	+	w	+
Fermentation of glucose with acid	+	+	+
Glucose with gas	-	+	-
Dulcitol	d	+	-
D-xylose	w	+	+
D-melobiose	-	+	-
Fructose	+	+	+
Galactose	+	+	D
L-Arabinose	-	+	-
L-sorbose	+	-	-
Lactose	-	+	-
Maltose	+	+	D
Mannitol	-	+	-
Raffinose	-	-	-
Rhmnose	-	+	-
Probable identity	*L. plantarum*	*L. brevis*	*L. delbrucki*

+, Positive reaction; -, negative reaction; w, weak reaction; D/d= variable.

Characterisation of isolates AS_1, AS_2 and KN_4

The morphological, physiological and biochemical characteristics as well as carbohydrate fermentation profile of the three selected LAB isolates (AS_1, AS_2 and KN_4) are shown in Table 1. Based on these biochemical data, isolates AS_1 was identified as *Lactobacillus plantarium,* AS_2 as *Lactobacillus brevis* and, KN_4 as *Lactobacillus delbruckii.*

Antagonistic activity of partially purified inhibitory compounds produced by isolates AS_1, AS_2 and KN_4

The antagonistic activities of the partially purified inhibitory compounds obtained from the isolates of LAB against the different target organisms based on agar well diffusion assay is shown in Table 2. The partially purified inhibitory compounds of isolates AS_1, AS_2 and KN_4 hadactivity against the different target organisms. The compounds inhibited the growth of the different organisms significantly ($P<0.05$). The three inhibitory compounds showed strong activity against *S. aureus*, *B. cereus* and *E. coli* ATCC 11775. The inhibitory compounds from isolates AS_1 and KN_4 were also active against *B. subtilis*. Only the inhibitory compound from isolate AS_1 affected *C. albicans* and *C. krusei*. The activities of all three inhibitory compounds were strongest against *S. aureus* ATCC 12600.

Total soluble protein in the partially purified inhibitory compounds

Partially purified inhibitory compounds produced by isolate AS_1 had 2.34 mg/ml protein while AS_2 and KN_4 had 1.6 and 2.13 mg/ml, respectively.

Effect of hydrolytic enzymes on stability of inhibitory compounds

The residual activity of the three inhibitory compounds

Table 2. Antagonistic activities (as zone of inhibition) of partially purified inhibitory compounds.

Target organism	Inhibition zone values (mm) mean ± S.D		
	AS_1	AS_2	KN_4
S. aureus ATCC 12600 (S1)	22.0±2.8	20.0±0.7	22.0±0.5
S. aureus (S2)	20.0±0.7	18.0±1.4	18.0±0.5
S. aureus (S3)	20.0±2.8	15.0±0.1	20.0±1.0
S. aureus (S4)	20.0±0.1	15.0±0.7	22.0±0.5
B. cereus (B1)	12.0±1.4	18.0±0.7	15.0±0.1
B. cereus (B2)	12.0±0.7	18.0±0.1	17.0±0.1
B. sublitis	15.0±1.4	0	16.0±0.7
E. coli ATCC 11775	18.0±0.7	15.0±1.4	18.0±1.0
Candida albicans (C1)	10.0±0.1	0	0
C. krusei	10.0±0.7	0	0

0 = No antagonistic activity detected.

Table 3. Residual activity of compounds after enzyme treatment.

Enzyme	Inhibition zone values (mm) mean ± S.D		
	AS_1	AS_2	KN_4
Trypsin	0	0	0
Pepsin	0	0	0
α-Amylase	22.0±2.9	20.0±0.9	22.0±0.6
Catalase	22.0±2.6	20.0±1.1	22.0±0.2
Control	22.0±2.8	20.0±0.7	22.0±0.5

0 = No activity detected.

was completely destroyed after treatment with proteolytic enzymes trypsin and pepsin. The activities were however, not affected by either α-amylase or catalase (Table 3).

Effect of heating on the activity inhibitory compounds

Results obtained after the partially purified inhibitory compounds heated at different temperatures indicate that these compounds were thermostable. There was no reduction in the residual activities of the various compounds, after heat treatment at 80°C for 30 min. However, thermostability of the compounds declined as the heating temperature was increased to 100°C for up to 15 min, and the variations in heat stability were significant ($P<0.05$). The activity of inhibitory compound produced by AS_2 was lost completely after heat treatment at 100°C for 15 min, while there was up to 60% reduction in the activity of the inhibitory compound from KN_4 after heating at 100°C for 15 min. The inhibitory compound obtained from AS_2 was the most stable to heat treatment, maintaining 100% activity after 15 min at 100°C.

Effect of pH on stability of inhibitory compounds

Each inhibitory compound was assayed for activity after 1 h incubation at different pH levels (pH 2.0-10.0). Data obtained indicates that pH affected the stability of the various inhibitory compounds differently (Table 4). The inhibitory compounds were shown to be stable between pH 5.0 and 8.0, (when activity at pH 7.0 was used as reference). However, there was approximately 33.4% decrease in the residual activity of the inhibitory compound produced by isolates KN_4 following treatment at pH 3.0, 4.0 and 9.0. Activity of this compound was completely destroyed following treatment at pH 2.0 and 10.0. Similarly, there was 40% reduction in the residual activity of inhibitory compound produced by isolates AS_2 following treatment at pH 4.0, while complete inactivation occurred at pH 2.0, 3.0, 9.0 and 10.0. The inhibitory compound produced by isolate AS_1, was stable between pH 3.0 to 8.0. Slight decrease in activity was detected at pH 2.0, 9.0 and 10.0. However, this decrease was not significant ($P>0.05$). Analysis of variance data confirm that stability of the inhibitory compounds produced by isolates AS_2 and KN_4 depended significantly on hydrogen ion concentration ($P<0.05$) as shown in Table 4.

DISCUSSION

This study was intended to isolate bacteriocin producing LAB from fermented traditional cereal based non-alcoholic beverages, and to test the capacity of partially

Table 4. The effect of pH on the stability of inhibitory compounds.

pH	Inhibition zone value (mm) mean ±S.D		
	AS_1	AS_2	KN_4
2.0	19.0±0.7	0	0
3.0	20.0±0.4	0	19.0±1.4
4.0	22.0±0.7	17.0±0.7	20.0±0.9
5.0	22.0±1.4	20.0±0.7	22.0±0.7
6.0	22.0±2.8	20.0±1.4	22.0 ±0.7
7.0 (control)	22.0±2.8	20.0±0.7	22.0±0.7
8.0	22.0±1.4	20.0±0.7	22.0±1.4
9.0	19.0±0.7	0	18.0±1.4
10.0	18.0±0.7	0	0

purified bacteriocins to antagonize food spoilage and pathogenic micro-organisms. This should serve as guide to the potential applicability of LAB and their elaborated inhibitory compounds in commercial biopreservation of foods, particularly those traditionally produced, improved or preserved using lactic fermentations. This should help base the application of these organisms in food presservation on empirical knowledge. The population of LAB on the tested foods was high. Kunun-zaki and akamu had populations of the order of 10^9 and 10^{10} cfu/g/ml, respectively. This is understandable considering that these beverages had undergone lactic fermentation as principal production step. Some strains produced antimicrobial principles that were shown to be bacteriocin.

The high incidence of bacteriocin producing LAB in these foods suggests that they may represent abundant source of potentially useful bacteria. A similar observation was made by Hartnett et al. (2002) in raw and malted cereals. LAB is also abundant contaminants in many other foods such as milk, meat and vegetables (Onda et al., 2002; Kalalou et al., 2004). The high incidence of LAB, including bacteriocinogenic strains in cereals has significant implications for the quality, safety and shelf life of these and related foods.

LAB have been reported to out-compete, and so inhibit other bacterial contaminants, resulting in improved quality and extended shelf life of products (Deegan et al., 2006). Many traditional African foods/ beverages including akamu and kunun-zaki are produced by fermention using LAB suggesting that the processes may result in improved quality and shelf life for food which may otherwise be plagued by problems such as inconsistent quality, poor hygiene and early spoilage. It was observed that partially purified bacteriocins produced by LAB isolates in this study inhibited *S. aureus, B. cereus, B. subtilis* and *E. coli.* The activities of the three inhibitory compounds were strongest against *S. aureus* ATCC 12600.

This is consistent with results reported for other bacteriocins (Schillinger and Lucke, 1987; De Martinis et al., 2001; Ogunbanwo et al., 2004). Tagg et al. (1976) suggested that bacteriocins usually have a narrow spectrum of activity inhibiting mostly Gram positive bacteria especially those closely related to the producer organism. The compounds produced by our isolates were also shown to be active against *E. coli* ATCC 11775. This observation is at variance with some earlier works that reported activity against only Gram positive organisms (Tagg et al., 1976; Schillinger and Lucke, 1987; Stevens et al., 1991). However, a few bacteriocins from LAB with activity against Gram-negative bacteria have also been reported. For examples, bacteriocin produced by strains of *Lactobacillus plantarum*, *Lactobacillus pentosus*, *Lactobacillus rhamnosus* and *Lactobacillus paracasei* isolated from boza, a Bulgarian traditional cereal beverage have been reported to be active against *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *E. coli* (Todorov and Dicks, 2006). Taken together, these results suggest that bacteriocins have a broader spectrum of activity than was previously reported and can inhibit even unrelated organisms.

The bacteriocin produced by isolate AS_1 in this study was active against *C. albicans* and *C. krusei*. This is potentially important for the exploitation of this isolate for food application. Although there are many reports on the production of antibacterial compounds by LAB (Megnusson and Schnurer, 2001; Lavermicocca et al., 2001), reports on bacteriocin inhibition of yeasts are comparatively few (Strom et al., 2002). Loss of antimicrobial property was observed after treatment of partially purified bacteriocin with proteolytic enzymes trypsin and pepsin, confirming the proteinacious nature of the active compounds. Similar observation was reported by Hartnett et al. (2002). Treatment of inhibitory compounds with catalase and α-amylase did not alter their activity, indicating that the inhibition recorded was not due to hydrogen peroxide and also that carbohydrate moieties if they exist in bacteriocins were not required for activity. Bacteriocins that do not contain or require carbohydrate for activity are classified into group I and II bacteriocins. Similar results were reported by Parente et al. (1996) and Todorov and Dick (2006). However, some researchers have reported slight inactivation by α-amylase for group IV bacteriocins. Such bacteriocins contain

a carbohydrate or lipid moiety that is necessary for activity (Klaenhammer, 1988; Shehane and Sizomore, 2002).The inhibitory compounds were shown to be stable over a broad pH range (pH 4.0 to 8.0) at room temperature. Similar observations on pH stability of bacteriocin have been reported (Hartnett et al., 2002; Shehane and Sizemore, 2002). The range of pH stability of these compounds is interesting in that, closely approximating the pH of lactic fermented foods, it can be expected to contribute to the safety and keeping quality of such foods. (Kunu-Nzaki and akamu have pH values in the range of 3.5 to 5.5 in this study). The inhibitory compounds were considerably heat stable, surviving treatment at 80°C for 30 min. Thermostability of bacteriocins have earlier been reported and been attributed to their small molecular sizes (Barefoot et al., 1992; Shehane and Sizemore, 2002). The thermostability of bacteriocins is of significance in their application in food systems, particularly if they are to be used together with pasteurisation in a multiple-hurdle approach to food preservation. However, the tolerance of bacteriocins to heat may be dependent on factors such as the level of purification, pH and other protective components (Barefoot et al., 1992) and these must be taken into account in designing applications for them in food. Based on standard microbiological and biochemical tests, the bacteriocinogenic isolates were tentatively identified.

The observed characteristics of the isolates were consistent with Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) descriptions for LAB. The three isolates obtained from the present study showed great potential for exploitation in food preservation. They were identified as *L. plantarum* (AS_1), *L. brevis* (AS_2) and *L. delbruckii* (KN_4). *L. brevis* has been reported to produce Brevicin, a bacteriocin which is active against *Listeria innocuna*, *L. grayi*, *L. monocytogenes*, *Strept. thermophilus* and *Enterococcus faecalis* (Teixeira, 1999). There is also a report on the extension of shelf life of fufu, a cassava based product by *L. brevis* (Ogunbanwo et al., 2004). *L. brevis* is used as starters in the production of many foods including pickled vegetables and rye breads and is now industrially available in freeze-dried form for use as starter culture. It is convenient and quick to use these cultures to make sourdough breads (Teixeira, 1999). *L. plantarum* produces plantaricin, which has been reported to inhibit beer spoilage LAB and is used in the brewing industry to prevent beer spoilage (Vaunghan et al., 2006). *Lactobacillus delbruckii* is utilized in the production of yoghurt. Incorporation of strains of *L. delbruckii* during fermentation increases the thickening properties which are necessary in yoghurt with low or no fat (Teixeira, 1999).

Conclusion

The three bacteriocin producing isolates of lactic acid obtained from the present study show great potential for exploitation in food preservation. It is conceivable that they and similar LAB may find application in African and related fermented foods where long shelf life products may be achieved by selection of appropriate bacteriocinogenic LAB that produce preservatives *in situ* in the fermented products.

Conflict of Interests

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

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Energetic assessment of soybean biodiesel obtainment in West Paraná, Brasil

Josefa Moreno Delai*, Jair Antonio Cruz Siqueira, Carlos Eduardo Nogueira, Reginaldo Ferreira Santos and Darlisson Bentes dos Santos

UNIOESTE, Western Paraná State University, Post graduation Program, Master Course of Energy in Agriculture, RuaUniversitária, 2069, CEP: 85.819-130, Jardim Universitário, Cascavel, Paraná, Brasil.

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This work presents the result of a research that aimed to assess soybean-based biodiesel production in the West region of Paraná State, showing that the growing usage of these fuels happens due to the need for alternatives to the use of fossil fuels, once biomass-based fuels have been an environmentally-friendly energetic alternative. The methodology consisted of determining the energetic consumption of biodiesel production. Energetic consumption was performed by considering the stages involved in soybean farming, oil extraction and production of pure biodiesel (B100); results were presented in megajoules (MJ). The energetic outputs obtained show that the energetic inputs in the farming stage totalized 2,411.53 MJ. Energetic outputs added up to 3,003.75 MJ and energy balance was 57,132.54 MJ. In the oil extraction stage, energetic inputs corresponded to a total of 16.80 MJ and energetic outputs to 17.29 MJ. Energetic balance presented a total of 5.14 MJ. In the soybean biodiesel production stage, energetic input was 59.06 MJ and energetic output, 39.69 MJ. Energetic balance corresponded to 33.26 MJ. The highest energetic consumption for soybean biodiesel production, contemplating all three stages, occurred in the farming stage, with 76% of the total energetic consumption, followed by energetic consumption in the production stage, with 21% of the total consumption.

Key words: Soybean production, energetic consumption, soybean biodiesel.

INTRODUCTION

The Brazilian energetic matrix presents the largest index of renewable sources in the world. According to the Ministry of Mines and Energy (MME, 2008), the internal offer of energy for 2007 was 45.9%, regarding renewable sources. However, non-renewable sources showed internal offer of 54.1% in the same year. The increase in the usage of such fuels mainly happens due to the need for alternatives to fossil fuels. Rocha and Neto (2007) states that the use of biomass-based fuels has been an environmentally friendly, or at least less impactful, and alternative. Biodiesel is produced from feedstock such as vegetable oils, animal fat, residual frying oil and fatty materials with high acidity Knothe et al. (2006). The main feedstocks for biodiesel production in Brazil are

*Corresponding author. E-mail: jodelai@fag.edu.br.

Table 1. Energy expenditure for different types of work.

Work type	Energy expenditure
Tractor, harvester and truck driving	3/6 of the EER
Sowing and fertilization	5/6 of the EER
Cover fertilization	6/6 ofthe EER
Transport of seeds and fertilizers	7/6 ofthe EER
Limestoneapplication	8/6 ofthe EER
Manual weeding	9/6 ofthe EER
Animal Tractionweeding	14/6 ofthe EER

Source: Bueno (2002). EER, Energetic expenditure during rest.

respectively: soybean (which represents 90% of the Brazilian production of vegetable oils), corn, sunflower, peanuts, cotton, canola, castor beans, babassu, palm and macauba. It is important to highlight that many crops in Brazil still have an extractive character, that is, do not have a competitive technology in their production system (Paulillo et al., 2007).

However, one may notice that there are many challenges when trying to boost biodiesel production in Brazil, especially the need for more researches, due to biodiesel's feedstock diversity, processes and usages. In that sense, the energetic analysis of biodiesel production may contribute as a tool to a formulation of environmental and technical-economic feasibility indicators in the comparison between oilseeds, as a way of diagnosing the best kind of crop for biodiesel production.

The objective of this study was to assess the energetic consumption in the production of biodiesel in the city of Cascavel, located in the west region of Paraná State, Brasil.

MATERIALS AND METHODS

This study was carried out in the municipality of Cascavel, in the west region of Paraná State, Brasil. The research took place at the Center of Development and Diffusion of technologies (CEDETEC, Portuguese acronym), at Faculdade Assis Gurgacz's (FAG) School Farm, in the period from November 2011 to July 2012. The CEDETEC works with research in the areas of production of biodiesel using vegetable oils and animal fats, technical feasibility, as well as performance testing of engines with different compositions of biodiesel.

Energetic balance of soybean biodiesel production

In order to determine the energetic balance of the soybean biodiesel, the study was divided into three stages: soybean farming, oil extraction and biodiesel production. In the farming stage, the objective was to search for the energetic consumption of one hectare of soybean. Cultural efficiency was performed according to Bueno (2002), who used two measures to express their results when working on an energetic analysis of a corn crop: cultural efficiency (equation 1) and liquid cultural energy (equation 2).

$$Cultural\ Efficiency = \frac{Useful\ \ Outputs}{Cultural\ \ Inputs} \quad (1)$$

$$Liquid\ Cultural\ Energy = \frac{Useful\ \ Outputs}{Cultural\ \ Inputs} \quad (2)$$

In order to calculate the energetic balance and energetic efficiency, the methodology suggested by Risoud (1999) was adopted. The same measures that capture the use of renewable energies were considered, as in equations 3 and 4.

$$\mathrm{EB} = \sum \mathrm{PGE} - \sum \mathrm{NREI} \quad (3)$$

$$\mathrm{EEF} = \frac{\sum \mathrm{PGE}}{\sum \mathrm{NREI}} \quad (4)$$

Where, EB is the Energetic Balance (MJ); PGE is the Products' Gross Energy (MJ); $NREI$ is the Non-Renewable Energy Inputs (MJ) and; EEF is the Energetic Efficiency.

In order to extract soybean oil, the energetic consumption was measured for the milling of 1 kg of soybean grains. The production stage considered the energetic consumption expended in the production of 1 L of soybean biodiesel.

Farming production

Determination of consumed energy by labor

To determine the consumed energy in the farming stage, the energetic consumption of the soybean crop was determined, considering energetic consumptions with labor, seeds, diesel oil, lubricant, grease, machinery and implements, fertilizers and agricultural defensives. As for the determination of daily consumptions, a sum of the activities in three periods of time was performed, according to the occupancy mode in number of hours for: resting time, working time, and non-professional occupation time (meals, hygiene, leisure and travel), by using the methodology applied by Risoud (1999) and Campos (2001). In order to calculate the energy expended with labor by human work, the methodology proposed by Mahan and Escott-Stump (1998) was used. As for the determination of the energetic consumption during rest, data for gender, weight, height and age were identified and associated to the developed operations, as shown in equations 5 and 6.

$$EER_M = 66.5 + 13.75 \times W + 5.0 \times H - 6.78 \times A \quad (5)$$

$$EER_F = 66.5 + 9.56 \times W + 1.85 \times H - 4.68 \times A \quad (6)$$

Where, EER_M is the Male Energetic Expenditure during Rest (MJ); EER_F is the Female Energetic Expenditure during Rest (MJ); W is the Weight (kg); H is the height (cm) and; A is the age, in complete years.

According to the methodology proposed by Carvalho (1974), all of the following were established: one third of the energetic expenditure during rest (EER), the fraction related to sleeping time, and half of the EER to the non-professional occupations. As seen in Carvalho (1974), and adapted by Bueno (2002), The determination of the EER related to working time was calculated based on the type of work performed by the farmer, as shown in Table 1. In order to calculate EER, the following were taken into account: H(cm), W(kg) and A(complete years). Labor was used in the farming stage, being one tractorist and common labor. The actions were divided into sowing and fertilization, application of pesticides and harvest.

Fuel, lubricant oil and grease

In order to calculate diesel oil's calorific power, the value 40.88 $MJ.L^{-1}$ was considered; as for lubricant oils, the value was 37.75

Table 2. Machines and equipments.

Equipments/Features	Life cycle (Years)	Annual use (Hours)
Massey Ferguson tractor, model MF 283(4X2 TDA), power 63.2 kW (86 cv) in the engine, board weight 2,850 kg (3,431 kg with ballast), front tires 12.4-24 R1 (39 kg) and rear tires 18.4-30 R1 (83 kg), used to perform pulverization and raw material transportation.	10	1000
Bar pulverizer by Jacto, model Condor, assembled, capacity 600 L, weight 400 kg, bar length 12 m, with 24 beaks, fan type, model DG 110-03, with spacings of 0.50 m.	10	480
Self-propelled grain cart by Massey Ferguson, model MF 3640, power 95.6kW (130 cv) in the engine, board weight 6,760 kg (7,193 kg with reel cut platform), front tires 23.1-30 R1 (138 kg) and rear tires 14.9-24 R1 (59 kg).	15	480
Precision seeder–fertilizer, by TatuMarchesan, model PST^3, spacing 450 mm, weight 3,170 kg.	10	480

Source: Massey Ferguson Manual, (2012).

MJ.L^{-1} (Brasil, 2004). Energetic expenditure was obtained by means of the expenditure in operations by the energetic coefficient of each energetic outlay.

Machinery and implements

Operations involving energetic consumption were divided into three stages as follows: sowing and fertilization, application of pesticides and harvest. The Table 2 presents the machines and implements used for soybean farming with features, life cycle and hours of annual use. As for the calculation of energetic depreciation, the methodology proposed by Beber (1989) was applied according to equation 7.

$$ED = \frac{(M - 10\ \% \text{ x } M)}{(L_u \text{ x } T_u)} \quad 7$$

Where, ED is the Energetic Depreciation; M is the Machinery or implement mass (kg); L_u is the Machinery or implement life cycle (hours) and; T_u is the Usage time (hours).

Regarding the calculation of energy used, the methodology proposed by Santos (2004) was applied, as follows in equation 8.

$$E_{mi} = \frac{ED}{EC} \quad 8$$

Where, E_{mi} is the Machinery and implements Energy (MJ); ED is the Energetic Depreciation (kg) and; EC is the Energetic Coefficient (MJ.kg^{-1}).

As for the sowing and fertilizing stage, the period of 1.5 h was considered; 0.5 h for pesticide application and 1.5 h for the harvest stage, according to data from FAG (2012). Energetic coefficients proposed by Comitre (1993) were used, considering the values of 14.62 and 13.012 MJ.kg^{-1}, respectively, for tractor and harvester. As for the energetic coefficients of implements and other equipments used in the operations until sowing, the value 8.62 MJ.kg^{-1} was adopted; regarding post-plantation operations using equipments, the value used was 8.35 MJ.kg^{-1}, as presented by the same author.

Fertilizers

In order to calculate energetic outlay with fertilizers, energetic coefficients presented by Bueno (2002) were used, considering for the soybean production system's energetic outlay coefficients 62.61, 9.63 and 9.21 MJ.kg^{-1}, respectively, for Nitrogen (N), Phosphorus (P_2O_5) and Potassium (K_2O). As for the determination of energetic consumption with fertilizers, the dosage of 300 kg.ha^{-1} of the formulation 02.20.20 was considered, according to data from FAG (2012), thus, for soybean cultivation, 6 kg.ha^{-1}of N, 60 kg.ha^{-1} of P and 60 kg.ha^{-1} of K were used.

Pesticides

Regarding pesticides, the energetic coefficients indicated by Pimentel (1980) were used, being 347.88 MJ kg^{-1}for herbicides; 311.07 MJkg^{-1} for insecticides; and 216.03 MJkg^{-1}for fungicides. Both herbicides and pesticides were applied twice; insecticides were applied once, according to data from FAG (2012). Quantities used were: 2.9 l ha^{-1} of herbicides, 0.643 l ha^{-1} of fungicides, and 0.30 l ha^{-1} of insecticides.

Seeds

In order to determine soybean seeds' energetic consumption, the present work referred to the methodology proposed by Pimentel (1980), who assigned the soybean seed to the energetic value that corresponds to the fossil energy applied to its production, measuring up to 16.736 MJ.ha^{-1}. During soybean sowing, the quantity of 50 kg.ha^{-1} seeds was considered, as found in data from FAG (2012).

Extraction stage

Data used for the determination of soybean oil extraction were obtained in FAG (2012); such data indicate that from 1 kg of soybean grains, one can obtain 13% of soybean oil, 81% of soybean bran and 6% of losses.

Soybean grain energetic expenditure

In this study soybean grains were considered as an energetic entrance in the soybean oil extraction process. Based on data from the oil extraction stage, it was possible to assess that 1 kg of soybean grains yielded 0.13 kg of soybean oil and 0.81 kg of

Table 3. Energetic coefficients of raw materials used in soybean biodiesel production.

Raw material	Unit	Energetic coefficient (MJ)
Firewood [1]	$MJ.kg^{-1}$	19.88
Glycerin [1]	$MJ.kg^{-1}$	32.81
Catalyst (NaOH) [1]	$MJ.kg^{-1}$	44.45
Methanol [2]	$MJ.kg^{-1}$	22.70
SoybeanOil [2]	$MJ.kg^{-1}$	47.80
Electric Energy [3]	$MJ.kWh^{-1}$	3.60
SoybeanBiodiesel [4]	$MJ.kg^{-1}$	39.11

Sources: [1] Bonometo (2009); [2] Sheehan and Camobreco, (1998); [3] Nogueira (1987); [4].

soybean bran. The calculation of the grain's energetic expenditure in the oil extraction process happened by multiplying the quantity of soybean grains used by the energetic coefficient, which was used for soybean grains of 16.80 $MJ.kg^{-1}$, as presented by Cavalett (2008).

Soybean oil energetic expenditure

As for the determination of the soybean oil energetic expenditure, it was necessary to consider that during the extraction stage, the value found was 0.13 kg of soybean oil per kilogram of soybean grains. The calculation of soybean oil energetic expenditure in the process was performed by multiplying the quantity of soybean oil used by the energetic coefficient, according to Cavalett (2008) is 39.60 $MJ.kg^{-1}$.

Soybean bran energetic expenditure

As for the determination of the soybean bran energetic expenditure, it was taken into account that during the extraction stage, the value found was 0.81 kg of soybean bran per kilogram of soybean grains. According to Mourad (2008), soybean bran's energetic coefficient is 15.00 $MJ.kg^{-1}$. Energetic consumption was determined from the product between the energetic coefficient and the quantity of soybean bran used in the extraction process.

Energetic consumption in biodiesel production

In order to determine energetic consumption, it is considered that for biodiesel production, the methylic route was used; as energetic inputs, the following were considered: firewood, catalyst, methanol, soybean oil and electric energy. Energetic outputs were glycerin and biodiesel. The coefficients for each raw material used in biodiesel production are represented in Table 3, according to values presented by Bonometo (2009); Sheehan and Camobreco, (1998); Nogueira (1987). Data for calculating energetic consumption were determined based on the production of 1 L of pure soybean biodiesel (B100), as seen in data from FAG (2012). The firewood used for biodiesel production was Bracatinga (*Mimossa scabrella*). Quantity used was 19.88 kg to produce 1 L of biodiesel. Energetic consumption was obtained by the product between the quantities of wood used in the process by the energetic coefficient. The NaOH was used as the catalyst for biodiesel production. The quantity used in the process was 0.055 kg of NaOH for the production of 1 L of biodiesel. The process of soybean biodiesel production happened by means of transesterification by methylic route, using 0.18 kg of methanol. In order to produce 1 L of soybean biodiesel, 0.9 kg of soybean oil was used.

In the process of producing 1 L of biodiesel, according to data from FAG's School Farm's Biodiesel laboratory, 0.045 kWh of electric energy was consumed. Electric energy was calculated by considering the energy consumed in function of the engines' power by the usage time of each engine during the process. During the process of producing 1 L of soybean biodiesel, according to data from FAG's School Farm's Biodiesel Laboratory, 0.0955 kg of glycerin was produced as sub product. Glycerin energetic value was calculated, considering it as an energetic output, by the product between the energetic coefficient and the quantity of glycerin generated in the production of 1 L of biodiesel. The pure biodiesel was considered, produced by means of the methylic route as an energetic output of the process. The liter of biodiesel produced corresponded to 0.955 kg of pure soybean biodiesel (B100).

RESULTS AND DISCUSSION

Energetic consumption in sowing and fertilization

In the operation of sowing and fertilization presented in Table 4, one may notice that the highest energetic consumption found during such operation was under indirect energy, due to the use of chemical fertilizers; such energy was responsible for more than 58% of the consumption. Jasper (2009), when analyzing a crambe crop, stated that chemical fertilizers present the highest caloric consumption, with more than 71% of indirect energy. Bueno (2002), in a study on a corn crop, found the following values: 28.31% for direct energy and 71.69% for indirect energy. Diesel oil, which is a fossil-based component of direct energy, stands out for its large participation in direct energy expended: 37.64%. Ferreira (2010), in a study carried out in the state of Rio Grande do Sul on the energetic and economic matrix of soybean crops, states that diesel oil represents 49% of the total consumption of direct energies.

Energetic consumption in harvest

Harvest operations significantly consume direct energy. Direct energy superiority (98.16%) occurs due to the broad use of fossil sources, particularly represented by the energetic expenditure with diesel oil. The value found

Table 4. Energy inputs in the operation of sowing and fertilization, MJ. ha^{-1}.

Type, source and method	Cultural inputs (MJ)	Participation (%)
Directenergy	**1.048.74**	**40.96**
Biological		
Labor		
Tractorist	9.54	0.37
Common	23.52	0.91
Seed	836.8	32.67
Fossil		
Diesel oil	168.92	6.59
Lubricant	3.02	0.11
Grease	6.94	0.27
Indirectenergy	**1.511.92**	**59.04**
Industrial		
Tractor	2.5	0.09
Seeder	3.36	0.13
Fertilizer	1.506.06	58.81
Total	**2,560.66**	**100**

by Jasper (2009) for direct energies in the energetic study of crambe was 96.46% of the total consumption. Bueno (2000) also found a close value for direct energies (90.37%) in a study on corn crops. The seed, component of the biologically based direct energy, stands out for its elevated participation in the expended direct energy, corresponding to 32.67%, followed by diesel oil caloric expenditure of 6.59%.

Energetic consumption in the application of pesticides

In the operation of pesticide application presented in Table 5, one can verify that the energetic consumption under indirect energy happens due to the use of herbicides, once this type of energy was responsible for more than 44.97% of the total consumption. Jasper (2009), when analyzing a crambe crop, also relates the herbicide to the highest energetic consumption, with 44.31%. In the indirect energy analysis, one may highlight the high value of the industrial energetic source represented by the harvester, which stands for 1.84% of the energy consumption.

Energetic consumption in soybean production

Energetic consumption in soybean production showed in Table 7 first presents sowing and fertilization consumption, with 47.20% of the total, followed by herbicides, fungicides and insecticides, with 45.01%. The operations which present the highest energetic consumption are sowing and fertilization, adding up to 47.20% of the total consumption.

Energetic balance in soybean production

In Table 8 one can find values for soybean production cultural efficiency, by means of the energetic outlay structure, in which energy inputs and outputs are quantified and presented in energetic units by relating soybean production to the technical schedule shown, considering an average productivity of 3,500 $kg.ha^{-1}$; total productivity was 58.35 sacks (60 kg per sacks) per hectare, what characterizes a built-in energy in production equal to 58,576.00 MJ. Direct energies represent 44.53% of the total energetic consumption and indirect energies represent a value which is somewhat over it, with 55.47% Bueno (2002), when studying a corn crop, found relative balance with 47.19% for direct energies and 52.81% for indirect energies. One may notice in Table 8 that the liquid cultural energy was 53,160.72 MJ, what led to a cultural efficiency of 10.82 MJ.

Energetic balance in soybean oil extraction

In Table 9, one can observe the results regarding inputs with 16.80 MJ of the energetic consumption of the total researched, as well as outputs with 17.29 MJ for the production of 1 kg of soybean grains. Serrão and Ocácia (2007) determined close values for energetic production of both soybean bran and oil, which were respectively 10.929 and 6.799 MJ. Energy balance followed Risoud's (1999) methodology, in which the sum of gross energy in the process is subtracted from the sum of non-renewable inputs. Once the gross energy used in the system was soybean oil and energy inputs were not considered in this process, it was possible to define the energetic balance value for soybean oil production, which was 5.148 MJ.

Table 5. Energy input, in MJ.ha^{-1}, in the operation of herbicide, fungicide and insecticide application.

Type, source and method	Cultural inputs (MJ)	Participation (%)
Direct energy	**957.55**	**42.66**
Biological		
Labor		
Tractorist	20.70	0.92
Common	42.45	1.89
Fossil		
Diesel oil	844.60	37.64
Lubricant	15.10	0.67
Grease	34.70	1.54
Indirectenergy	**1.286.22**	**57.34**
Industrial		
Tractor	37.5	1.67
Sprayer	7.65	0.34
Herbicide	1.008.85	44.97
Fungicide	138.9	6.20
Insecticide	93.32	4.16
Total	**2.243.77**	**100.00**

Table 6. Energy inputs, in MJ. ha^{-1}, participations in harvest.

Type, source and method	Cultural inputs (MJ)	Participation (%)
Directenergy	**414.81**	**98.16**
Biological		
Labor		
Tractorist	7.62	1.81
Common	27.44	6.5
Fossil		
Diesel oil	368.73	87.25
Lubricant	4.07	0.96
Grease	6.94	1.64
Indirectenergy	**7.79**	**1.84**
Industrial		
Harvester	7.79	1.84
TOTAL	**422.60**	**100**

Energetic balance in the production of soybean biodiesel

The analyzed soybean biodiesel was obtained in a methylic route (using methanol). Analyzed inputs were: electricity, firewood, catalyst, soybean oil and methanol. Energetic outputs were: soybean biodiesel and glycerin. Energy balance may be restricted to only one industrial stage of biodiesel production, in which the basic raw materials are: oil, alcohol, catalyst, electric energy and heat (Nogueira, 1987). Based on the raw materials assigned for the production of 1 L of soybean biodiesel, the energetic consumption for soybean biodiesel production was then determined, as shown in Table 10. The energy balance was characterized by the sum of gross energy in the process subtracted from the sum of

Table 7. Energetic consumption in soybean production under direct drilling system, MJ.ha^{-1}.

Operation	Energetic participation in the production system	
	MJ.ha^{-1}	Percentage (%)
Sowing and fertilization	2.560.66	47.20
Herbicide, fungicide and insecticide application	2.441.59	45.01
Harvest	422.604	7.79
Total	**5.424.854**	**100**

Table 8. Outlay structure by type, source and method; cultural inputs, useful outputs, liquid cultural energy, soybean production cultural efficiency, and energetic balance.

Type, source and method	Cultural inputs (MJ)	Participation (%)
Directenergy	**2.411.53**	**44.53**
Biological		
Labor		
Tractorist	37.86	0.70
Common	93.41	1.72
Seed	836.8	15.45
Fossil		
Diesel oil	1.382.25	25.53
Lubricant	19.29	0.36
Grease	41.91	0.77
Indirectenergy	**3.003.75**	**55.47**
Industrial		
Machinery and implements	58.8	1.09
Herbicide, fungicide and insecticide	1.438.89	26.57
Chemical fertilizers	1.506.06	27.81
Cultural inputs	**5.415.28**	**100.00**
Useful outputs	**58.576.00**	
Liquid cultural energy	**53.160.72**	
Cultural efficiency	**10.82**	
Energeticefficiency	**40.58**	
Energetic balance	**57.132.54**	

Table 9. Energetic balance in soybean production.

Raw materials	Quantity	Energetic coefficient (MJ)	Energetic production (MJ)
Input			**16.80**
Grains	1 kg	16.80	16.80
Output			**17.29**
Soybean oil	0.13 kg	39.60	5.148
Soybean bran	0.81 kg	15.00	12.15
Balance			**5.148**

Note: Energetic balance in soybean oil production.

Table10. Energetic balance of soybean biodiesel.

Raw material	Quantity	Energetic coefficient (MJ)	Energetic production (MJ)
Input			**59.06**
Firewood	0.47	19.88	9.34
Catalyst (NaOH)	0.05	44.45	2.44
Methanol	0.18	22.7	4.09
Soybean oil	0.9	47.8	43.02
Electric energy	0.04	3.6	0.16
Output			**39.69**
Glycerin	0.09	24.44	2.34
Soybean biodiesel	0.95	39.11	37.35
Balance			**33.26**

Note: Energetic balance of soybean Biodiesel.

Table 11. Energetic balance of the production of soybean biodiesel.

Stage	Energetic balance (MJ)	Energetic balance (MJ. Liter^{-1})
Soybean farming	57.132.54	121.96
Oil extraction	5.148	5.642
Biodiesel production	33.26	33.26
Total	**57,170.94**	**160.86**

Note: Energetic balance of each stage in the production of soybean biodiesel.

non-renewable inputs (Risoud, 1999). By having soybean biodiesel as the system's gross energy and methanol as the source of non-renewable energies, it was possible to define the energetic balance for the production of soybean biodiesel, which was 33.26 MJ (Table 11). Pimentel and Patzeck (2005) estimated the energy consumption in the production of 1 ton of soybean biodiesel in the United States of America to be 19.78 MJ. Serão and Ocácia (2007), in a study on soybean biodiesel production in the state of Rio Grande do Sul, estimated the energy balance to be 39.38 MJ.

Energetic balance of each stage for the production of soybean biodiesel

In order to determine soybean biodiesel's energetic balance, it was necessary to use data from soybean production, soybean oil extraction and data from the production of biodiesel obtained in a methylic route, in which it was considered as the production of 1 ha of soybean, 1 kg of soybean grains and 1 L of soybean bio-diesel. The energetic balance of the stages of soybean biodiesel production presented a value of 57,170.94 MJ. Chechetto (2010), in a study on the energetic balance of castor bean biodiesel, found very similar values: 56,830.56 MJ. One must highlight that in the energetic consumption in the obtainment of 1 L of biodiesel, including the three stages, soybean farming presents the highest energetic consumption (76%), followed by energetic expenditure for soybean biodiesel production (21%) and the one with lesser consumption, soybean oil extraction (3%). It is also substantial to point out that the low energetic consumption in the extraction stage happens due to the calculation of energetic consumption being related only to main inputs and outputs (soybean grains, oil and bran), not concerning to other inputs and outputs, such as electric energy, labor, and others.

Conclusion

Based on the results obtained in the conditions in which this work was carried out, one can conclude that, the energetic inputs in the farming stage added up to 2,411.53 MJ. Energetic outputs totalized 3,003.75 MJ, and energy balance was 57,132.54 MJ. In the oil extraction stage, energetic inputs corresponded to 16.80 MJ and energetic outputs to 17.29 MJ. Energetic balance was 5.14 MJ. The soybean biodiesel production stage presented energetic inputs of 59.06 MJ and energetic outputs of 39.69 MJ. In this stage, energetic balance was 33.26 MJ. The highest energetic consumption for soybean biodiesel production, regarding all three stages, occurred in the farming stage, with 76% of the total energetic consumption, followed by energetic consumption in the production stage, with 21% of the total consumption.

Conflict of Interests

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

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Platelet function, anthropometric and metabolic variables in Nigerian type 2 diabetic patients

Jeje, S. O.[1,2]*, Shittu, S. T.[2] and Fasanmade, A. A.[2]

[1]Department of Human Physiology, Cross River University of Technology, Okuku Campus, Cross River State, Nigeria.
[2]Department of Physiology, University of Ibadan, Ibadan, Nigeria.

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This study examined the effects of anthropometric variables and metabolic imbalance on platelet aggregation in diabetic patients. A total of 109 volunteer were used; 58 diabetes mellitus (DM) patients (28 males and 30 females) who were receiving treatment at the University College Hospital Ibadan and 51 non diabetic control recruited from residents of Agbowo and Teachers of some secondary schools within the University of Ibadan. Body mass index (BMI) and body surface area (BSA) were assessed as indices of anthropometry, fasting blood sugar (FBS), plasma cholesterol and triglycerides (TAG) were determined using standard method and platelet aggregation test was done on the whole blood. Platelet aggregation ratio was higher in non diabetic compared to the diabetic subjects (P<0.001). The mean platelet aggregation ratio was also significantly higher in the male diabetic when compared to the female diabetic group (P<0.001). There was a significant linear relationship between platelet aggregation ratio and BMI (P<0.01), age (P0.05), FBS (P< 0.01), plasma cholesterol (P<0.01) and plasma TAG (P<0.05). However, the correlation coefficient between platelet aggregation ratio and BSA is not significant. In the non diabetic control subjects the correlation coefficient is not significant. Findings from this study suggest that, the increased platelet aggregation found in diabetic patients increased significantly with increased BMI but decrease with age. The mean platelet aggregation is also increased significantly with increase metabolic imbalance.

Key word: Platelet aggregation, anthropometry, diabetes mellitus, Body mass index (BMI), fasting blood sugar (FBS).

INTRODUCTION

Diabetes mellitus is a major degenerative disease in the world today (Ogbonnia et al., 2008). It is a serious lifelong condition that affects an estimated population of about 366 million adults, aged 20 to 79 years (IDF, 2011) and about 4.5% (14.7 million) of this population are in Africa. About 80% of this population remains undiagnosed (11.6

*Corresponding author. E-mail: dhikrilat@yahoo.com. Tel: +2348086327115.

Abbreviations: BMI, Body mass index; **BSA,** body surface area; **FBS,** fasting blood sugar; **TAG,** triglycerides.

million) (IDF, 2011). The high proportion of an undiagnosed population in Africa, may lead to an increased prevalence of complications. Studies on platelet aggregation in diabetes mellitus have presented conflicting results. While some authors reported normal aggregation (Heath, 1971; Perterson and Gornsen, 1978), increasingly more have reported platelet hyperaggregation (Szirites, 1970; Sagel et al., 1975; Colwell et al., 1976, Malik et al., 2012). The variations in the reports may be as a result of several factors which may include age. There is an increase in platelet aggregation in premenopausal women over the postmenopausal women in Caucasians (Mancini et al., 1965; Dacie et al., 1973).

The metabolic abnormalities that characterize diabetes such as hyperglycemia, increased free fatty acid and insulin resistance each provoke molecular mechanisms that alter platelet function and increase the production of several prothrombotic factors (Li et al., 2001; Mark et al., 2003; Asset et al., 2001). Evidence suggests that metabolic disorders frequently accompany excess body weight (Corsonello et al., 2003). Therefore, it is likely that BMI affects platelet aggregation in patients with diabetes mellitus. As far as we know no work has been done in this regard in Nigerian diabetic patients. Therefore, the aim of this study is to determine the effect of BMI, Age and BSA on platelet aggregation in African patients with diabetes mellitus.

MATERIALS AND METHODS

Study design and methods

The study was carried out on patients with diabetes mellitus attending the clinic at the University College Hospital Ibadan and Staff of the University of Ibadan and Abadina College University of Ibadan, who have volunteered to participate in the study. Ethical approval was granted from UI/UCH joint ethical committee (UI/UCH ethics committee assigned number: UI/EC/09/0101). The consent of the volunteers was obtained by a signed informed consent form. The procedure involved and the rationale behind the study was explained to the subjects. A total of 58 diabetic patients attending clinic at the University College Hospital Ibadan and 51 non diabetic control subjects from staff of University of Ibadan and Abadina College University of Ibadan were compared in this study. These include 28 males and 30 females, 25 males and 26 females for both diabetic patients and control subjects, respectively. The duration of the study was 8 weeks. The subjects were instructed to fast for about 12 h prior to the beginning of the study.

Determination of platelet aggregation ratio

Platelet aggregation test was done on the whole blood based on the principle of the methods of Wu and Hoak (Wu and Hoak, 1974). This test is based on the principle that circulatory platelet aggregates are fixed when exposed to a mixture of formalin and EDTA. The fixed platelet aggregates settle down on centrifugation, leaving a platelet rich plasma. The platelet aggregation ratio is 1 in the absence of aggregation (Ogunlade and Fasanmade, 2001). About 2 ml of blood was collected from an arm vein in the morning after overnight fast into a plastic syringe. This was gently dispensed into EDTA tube and EDTA/Formalin tube labelled according to their code. 0.1 ml of both EDTA and EDTA/Formalin sample was pipette to clean plain tubes and this was mixed with 1.9 ml of 1% ammonium oxalate, to allow it lyse the red blood cell. The samples were left on the laboratory table for 15 min at room temperature. Platelet count of samples in EDTA and EDTA/Formalin was manually determined using improved Neubauer counting chamber under light microscope. The platelets appeared under ordinary illumination as small (but not minute) highly refractive particles when viewed with the condenser racked down. The number of the platelets seen in an area of 1 mm^2 was counted and the number noted.

$$\text{Platelet aggregation ratio was calculated as: } \frac{\text{Platelet count in EDTA/Formalin}}{\text{Platelet count in EDTA}}$$

$$\text{Platelet aggregation is } \alpha = \frac{1}{\text{Platelet aggregation ratio}}$$

Determination of body mass index (BMI) and body surface area (BSA)

Weight and height were measured without shoes using standard weigh balance and standard meter rule and BMI and BSA were determined from the formula as given below:

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

Weight are measured in kg and height in meter

$$BSA = \frac{71.84 \times weight^{0.425} \times height^{0.725}}{10000}$$

Weight is measured in kg and height in cm.

Determination of FBS, plasma cholesterol and plasma TAG

Fasting blood sugar (FBS) was determined using one touch ultra-glucometer. The principle of which is based on the glucose oxidase method. Plasma cholesterol and plasma TAG were determined spectrophotometrically using Randox cholesterol and TAG kit (Randox laboratory UK).

RESULTS AND DISCUSSION

The mean platelet aggregation ratio was significantly higher in the nondiabetic group (0.84±0.02) when compared with the diabetic group (0.57± 0.02) ($p< 0.001$) (Figure 1). Also, the mean platelet aggregation ratio was significantly higher in the male diabetic (0.67±0.02) when compared with the female diabetic group (0.47±0.02) ($P< 0.001$) (Figure 2). In diabetic subjects there was a significant linear relationship between platelet aggregation ratio and age ($P<0.05$) as well as BMI

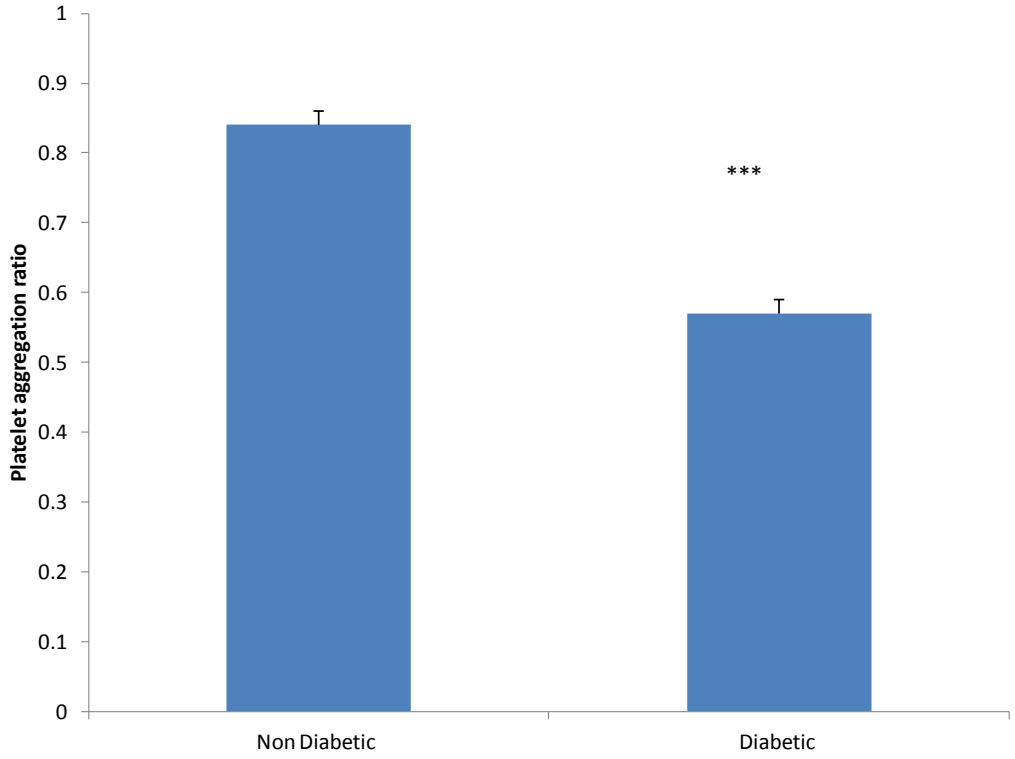

Figure 1. Comparison of mean platelet aggregation ratio between diabetes and non diabetes. N= non diabetes 51; diabetes 58; *** = P< 0.001.

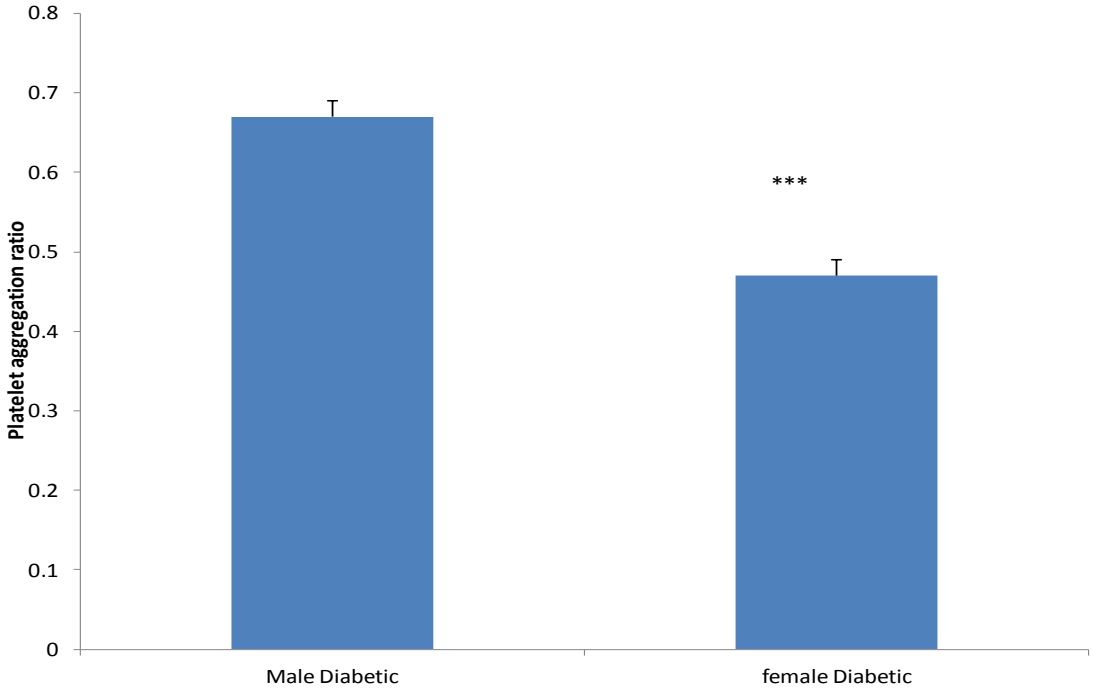

Figure 2. Comparison of mean platelet aggregation ratio between male and famale diabetic patient. N= male diabetic 28; female diabetic 30; *** = P< 0.001.

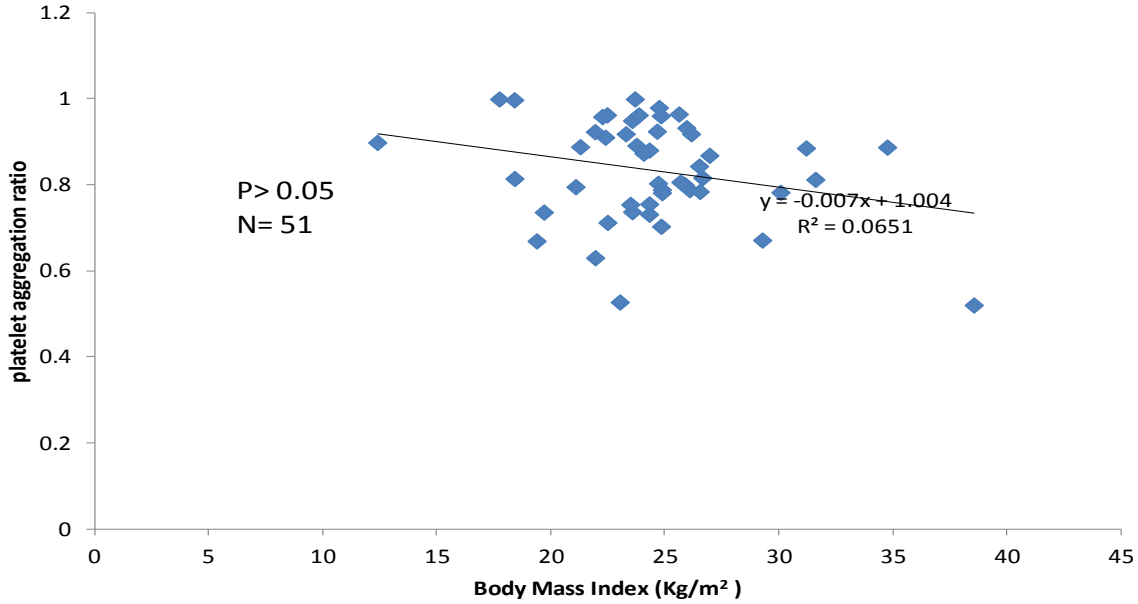

Figure 3. Scatter diagram showing correlation between platelet aggregation ration and BMI (Body Mass Index) in the control nondiabetic subject.

(P<0.01) (Figures 6 and 7). However, there was no significant association between platelet aggregation ratio and BSA (Figure 8). The correlation coefficient in the case of age was positive (0.307) and negative in the case of BMI (-0.395). There was also a significant linear association between platelets aggregation ratio and Plasma Cholesterol (P<0.01), Plasma TAG (P< 0.05) and Fasting Blood Sugar (FBS) (P<0.01) with platelet aggregation ratio (Figure 9 to 11). In the nondiabetic control group, the correlation coefficients between age, BMI and BSA with platelet aggregation ratio were not significant (Figures 3, 4 and 5). The correlation between plasma cholesterol, plasma TAG and FBS, with platelet aggregation ratio was however not significant in the non diabetic control group (Figure 12 to 14).

Findings obtained from this study confirm that platelet aggregation is significantly higher in diabetic compared to the non diabetic control subject. This is consistent with report from other populations and *in vitro* studies (Health et al., 1971; Sagel et al., 1975; Malik et al., 2012). Various mechanisms have been suggested to be responsible for this enhanced platelet activation and aggregation such as abnormal Ca^{2+}-ATPase activity (Rosado et al., 2004; Jardin et al., 2006), impaired Ca^{2+} homeostasis (Ishii et al., 1991), impairment in platelet signalling such as nitric oxide (NO) production (Trovati and Anfossi, 2002). However, *in vitro* studies by Kutti et al. (1986) showed no increase in adenosine diphosphate (ADP) sensitivity of platelet from diabetic patients. According to Malik et al. (2012), the reason for the contrasting result could be the fact that all the patients in the study by Kutti et al. (1986) were insulin dependent diabetics. And as described by Harrison and his colleague (1980), chronic use of insulin administration may restore prostacyclin PGI2 production in platelets of diabetic animals leading to a decrease in their aggregation tendency.

Results from this study also confirm that platelet aggregation in female diabetic patients is greater than the male diabetic patients. This is line with the findings of a study done by Kueh et al. (1982) who reported an increased platelet aggregation in female diabetics over the male diabetic group. The fact that diabetes increases the incidence of myocardial infarction, claudication and stroke more in women than in men with diabetes is well established in the literature (Kannel and McGee, 1985). These cardiovascular abnormalities may be secondary to platelet aggregation.

Diabetes mellitus is associated with increased occurrence of metabolic imbalance such as hyperglycemia, increased plasma cholesterol and triacylglycerol. This study showed in agreement that there is a positive correlation between these metabolic imbalance and platelet aggregation in African diabetic patients. A previous report by Valentovic and Lubawy (1985) showed that elevated glucose *in vivo* alters prostaglandin generation in rat platelets. Altered prostaglandin generation increases the aggregating capacity of platelets. In contrast, Malik et al. (2012) found no correlation between FBS, TAG and Total plasma

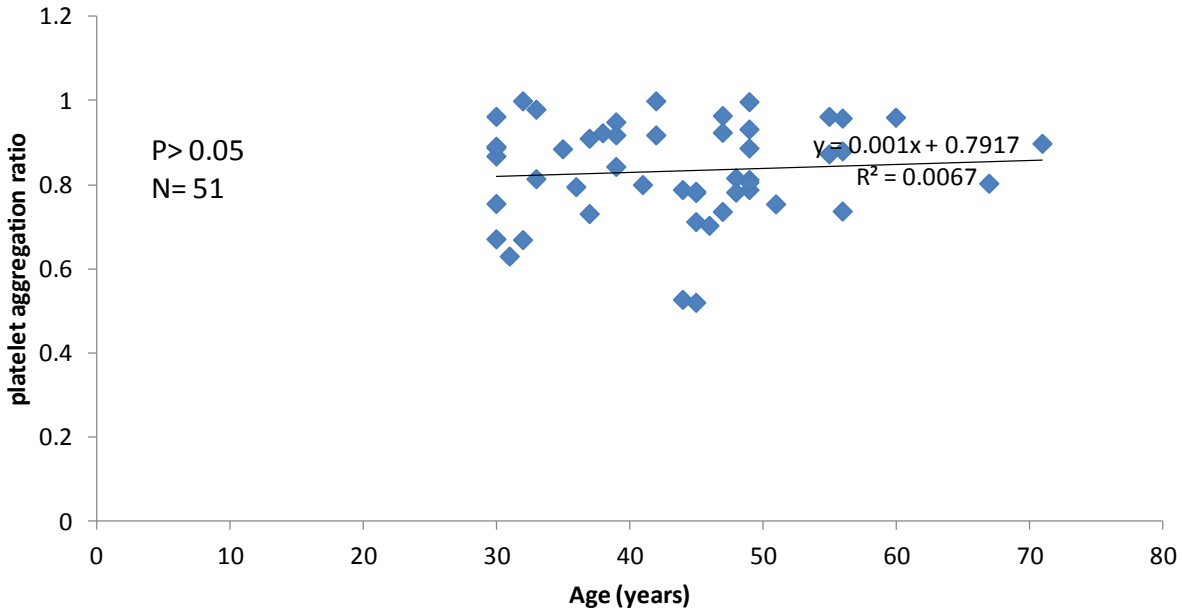

Figure 4. Scatter diagram showing correlation between platelet aggregation ration and age in the control non diabetic subject.

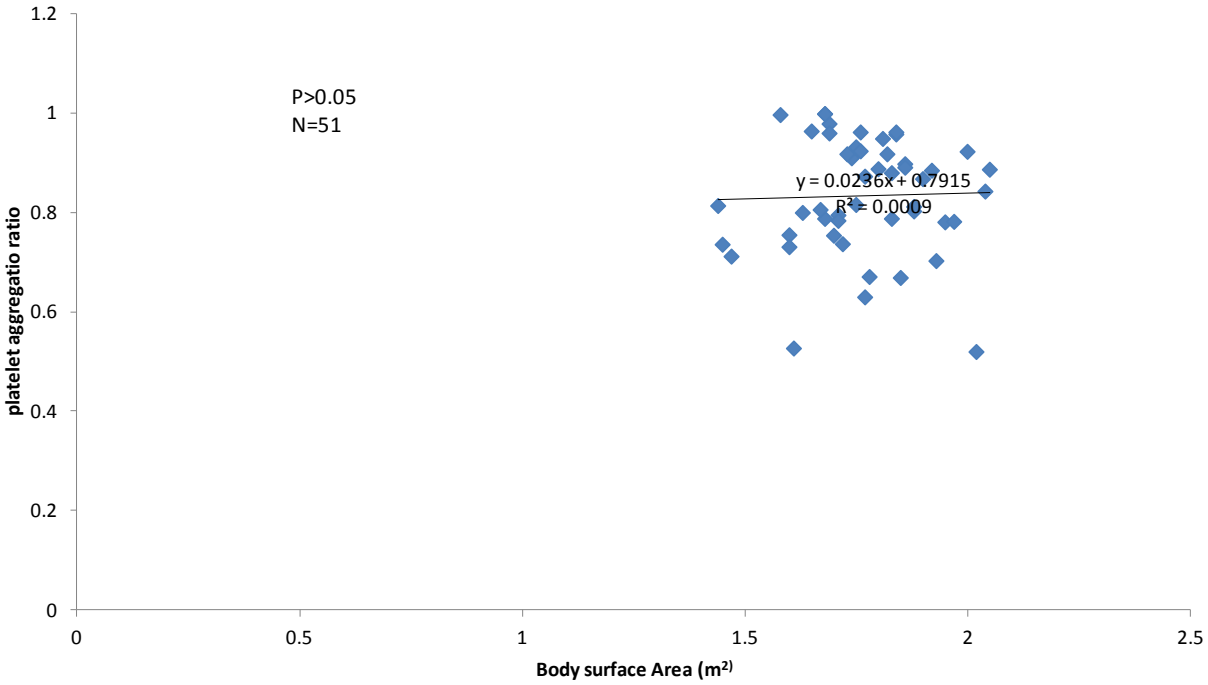

Figure 5. Scatter diagram showing correlation between platelet aggregation ration and BSA in the control non diabetic subject (body surface area).

cholesterol in Indians with early glucose intolerance or diabetic patients. The reason for this discrepancy is not known, but it may be due to geographical location, or the sample size, since our study comprise a larger sample size.

Another finding of this study is that anthropometric data

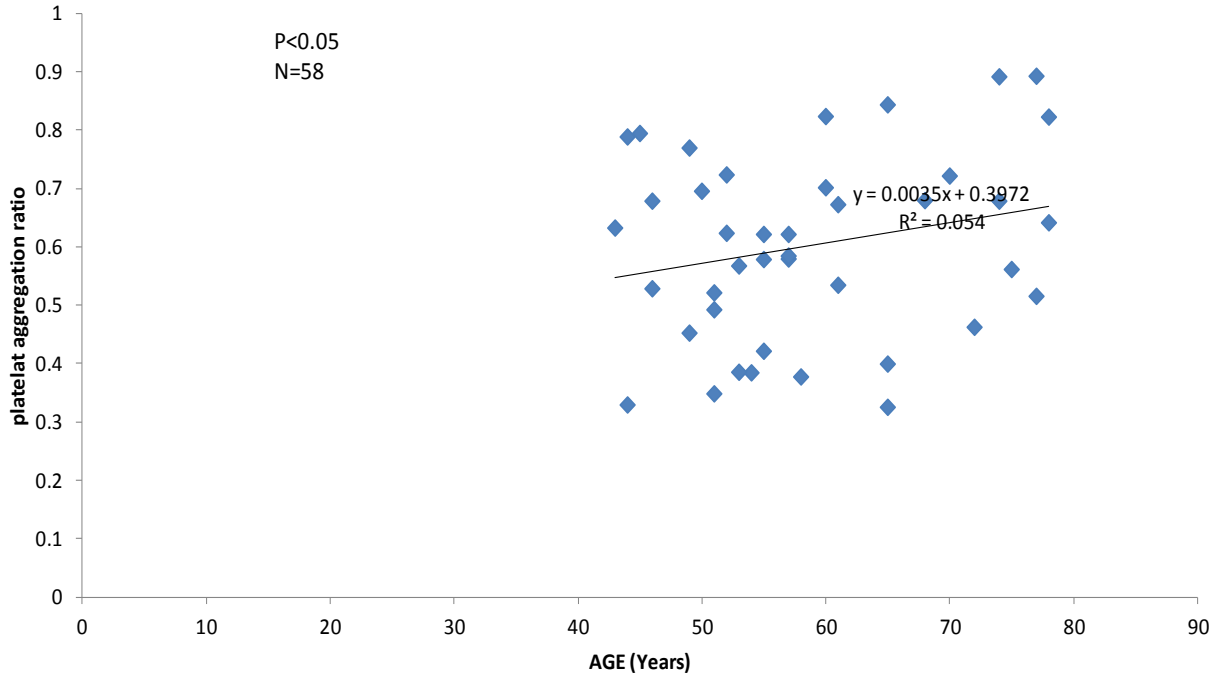

Figure 6. Scatter diagram showing correlation between platelet aggregation ration and age in the diabetic subject.

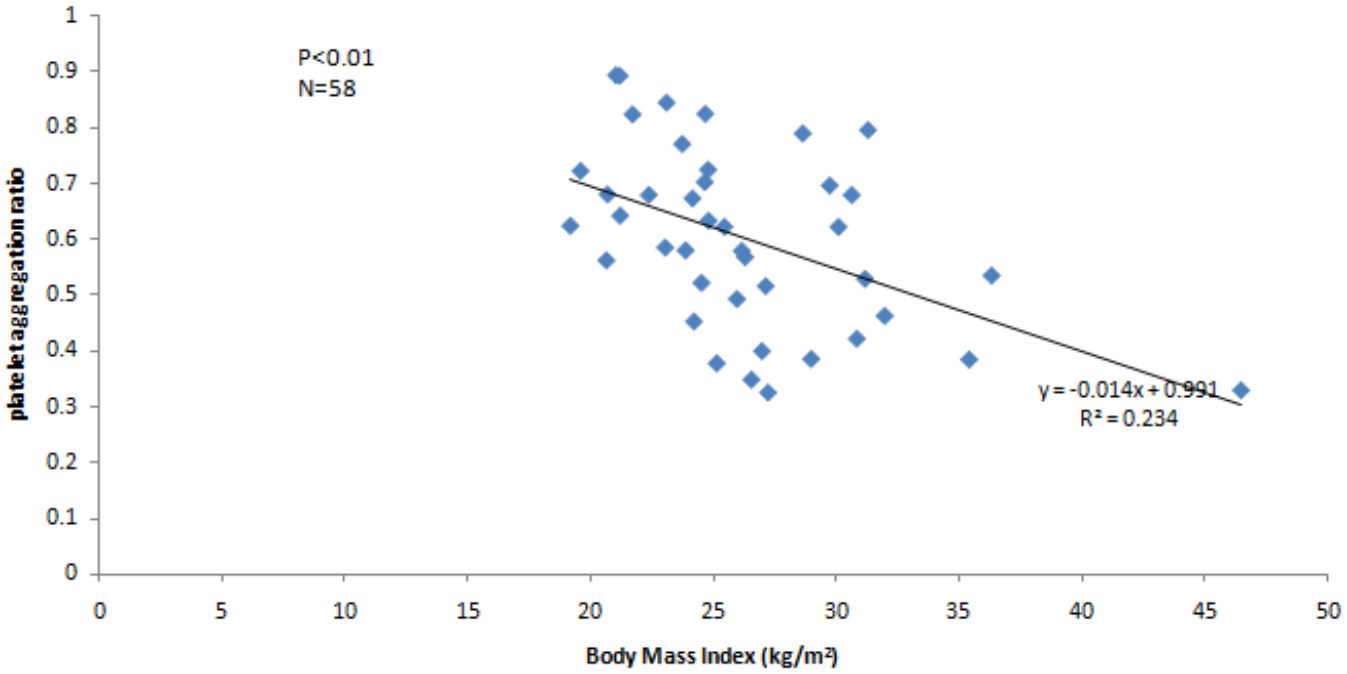

Figure 7: Scatter diagram showing correlation between platelet aggregation ratio and BMI (Body Mass Index) in the diabetic subject

such as Age and BMI correlate with increased platelet aggregation in diabetic patients. BMI increases in direct proportion with platelet aggregation. However, the correlation between Age and platelet aggregation is in inverse proportion. The reason for increased platelet aggregation in younger diabetic patients in this study is

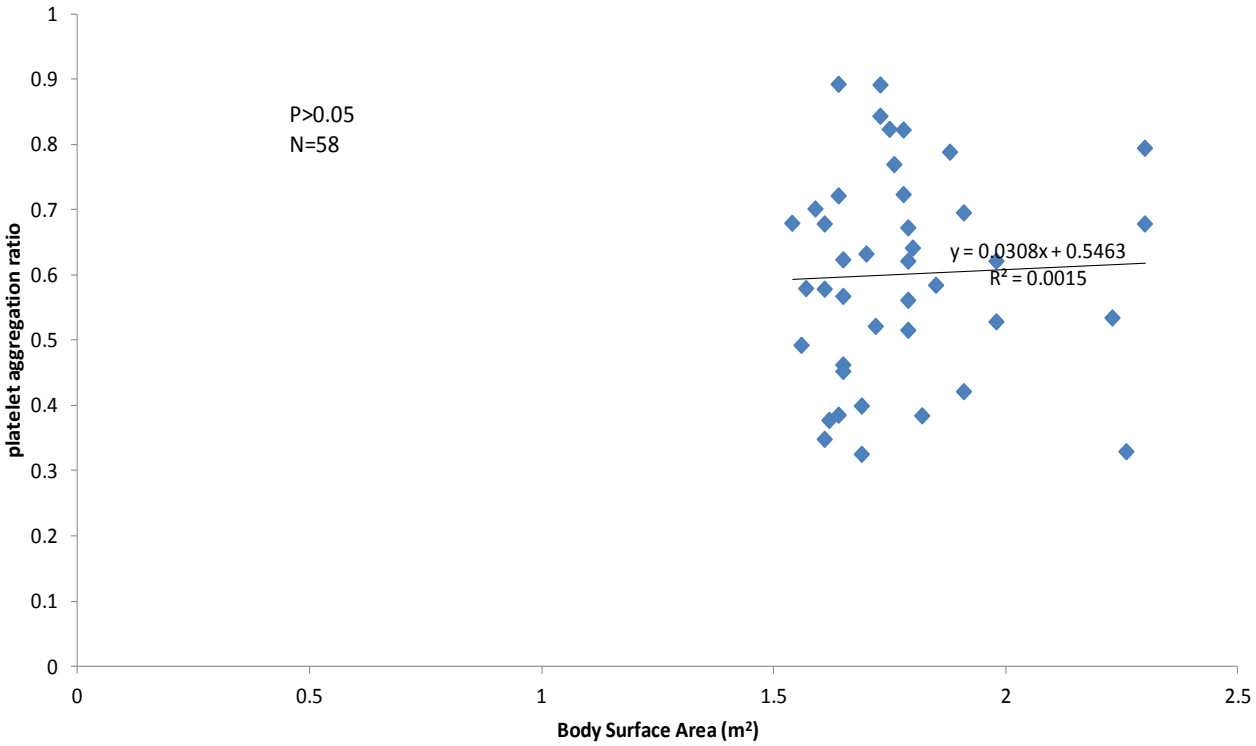

Figure 8. Scatter diagram showing correlation between platelet aggregation ration and BSA in the diabetic subject (body surface area).

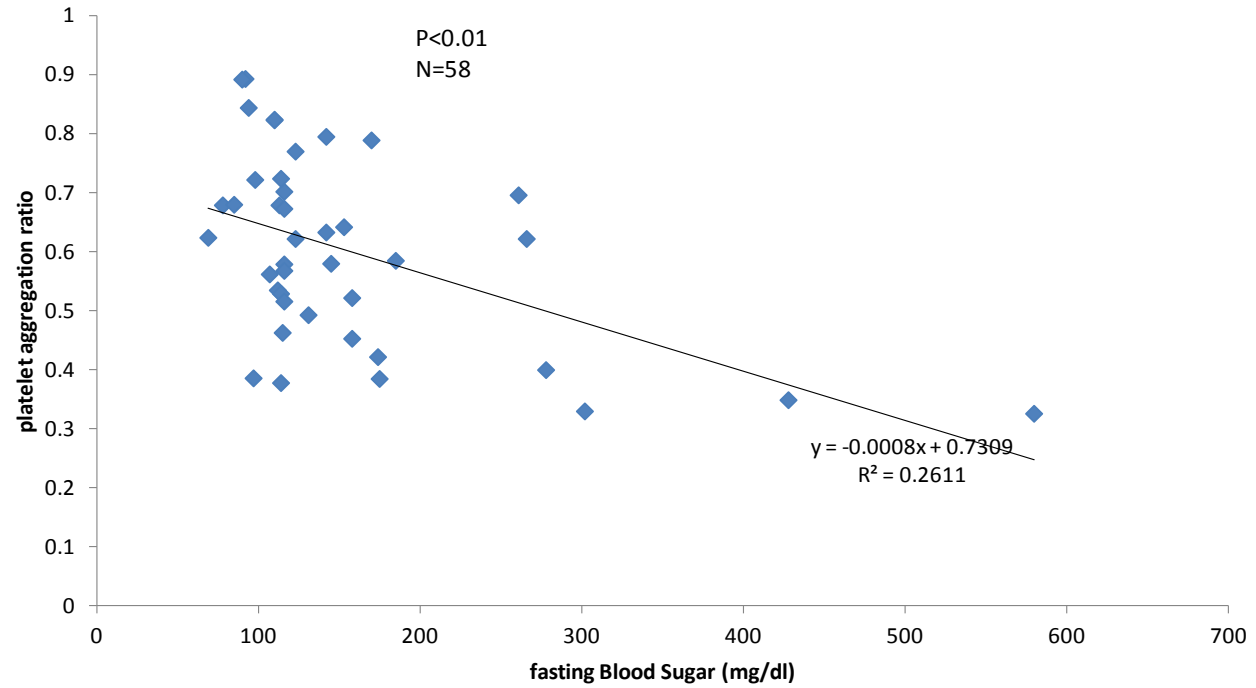

Figure 9. Scatter diagram showing correlation between platelet aggregation ration and FBS in the diabetic subject (Fasting blood sugar).

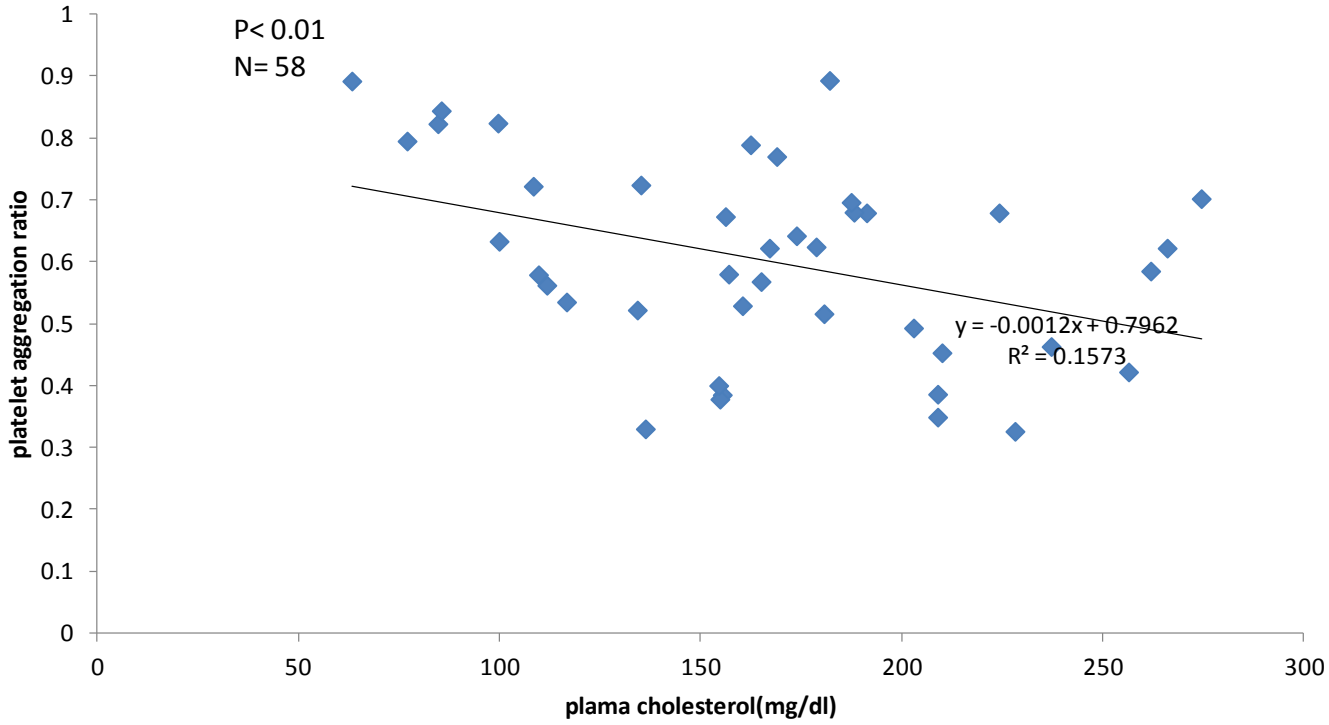

Figure 10. Scatter diagram showing correlation between platelet aggregation ration and plasma cholesterol in the diabetic subject.

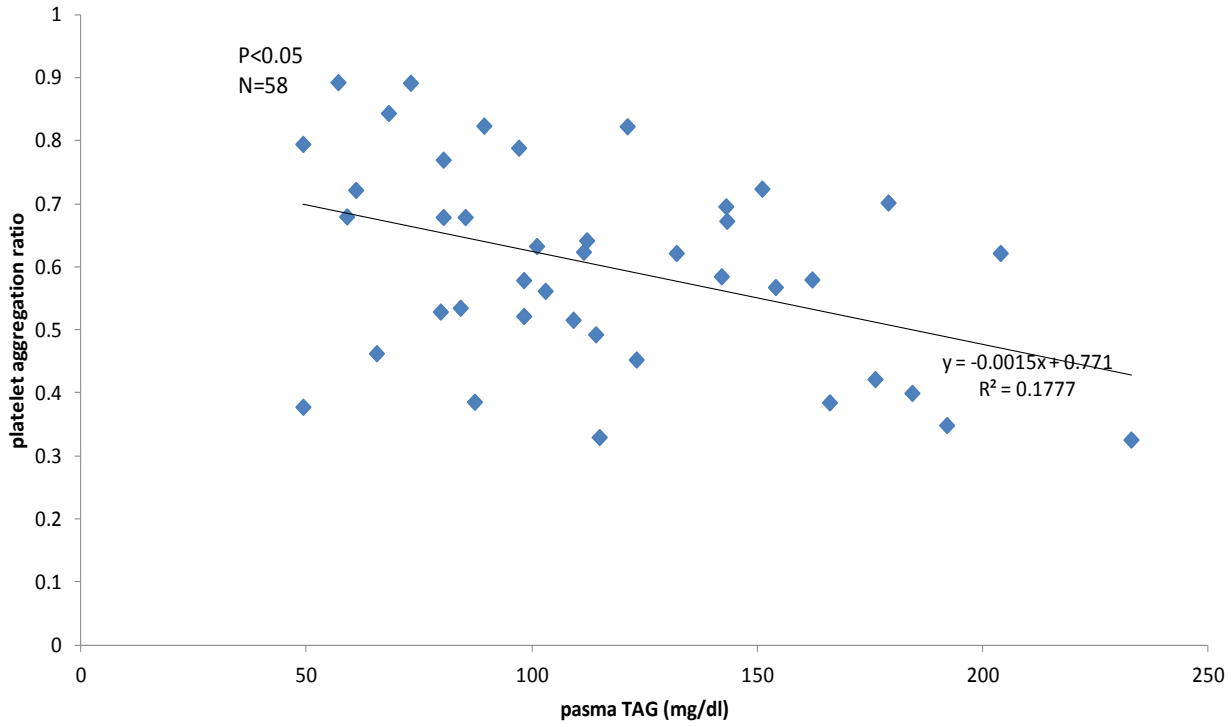

Figure 11. Scatter diagram showing correlation between platelet aggregation ration and plasma TAG in the diabetic subject (Triglyceride).

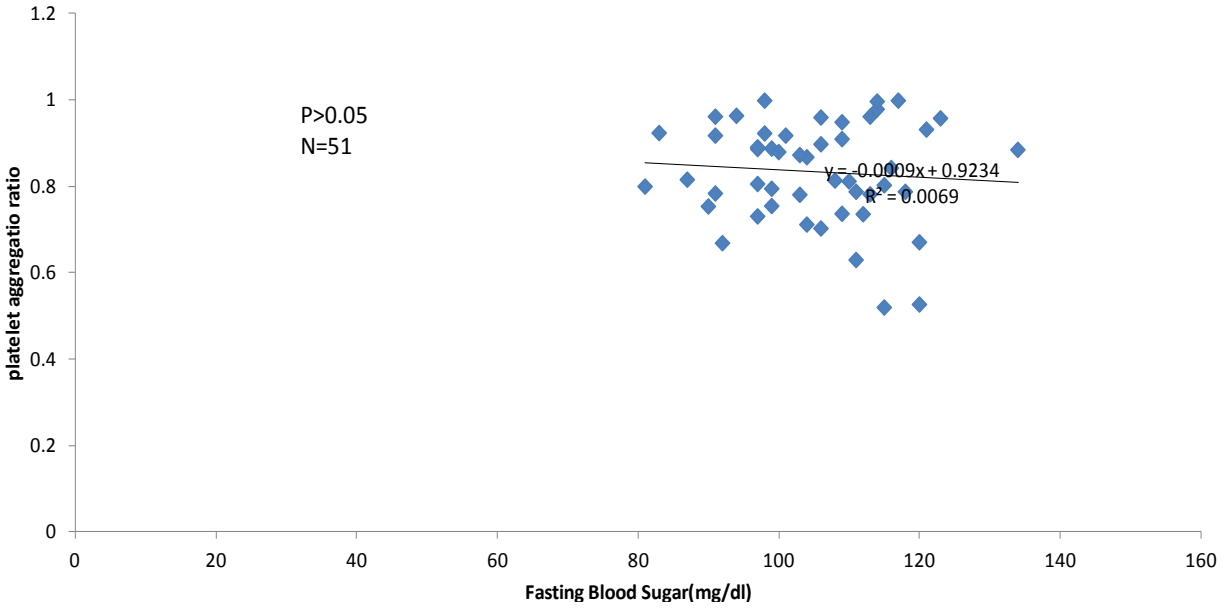

Figure 12. Scatter diagram showing correlation between platelet aggregation ration and FBS in the control non diabetic subject (Fasting blood sugar).

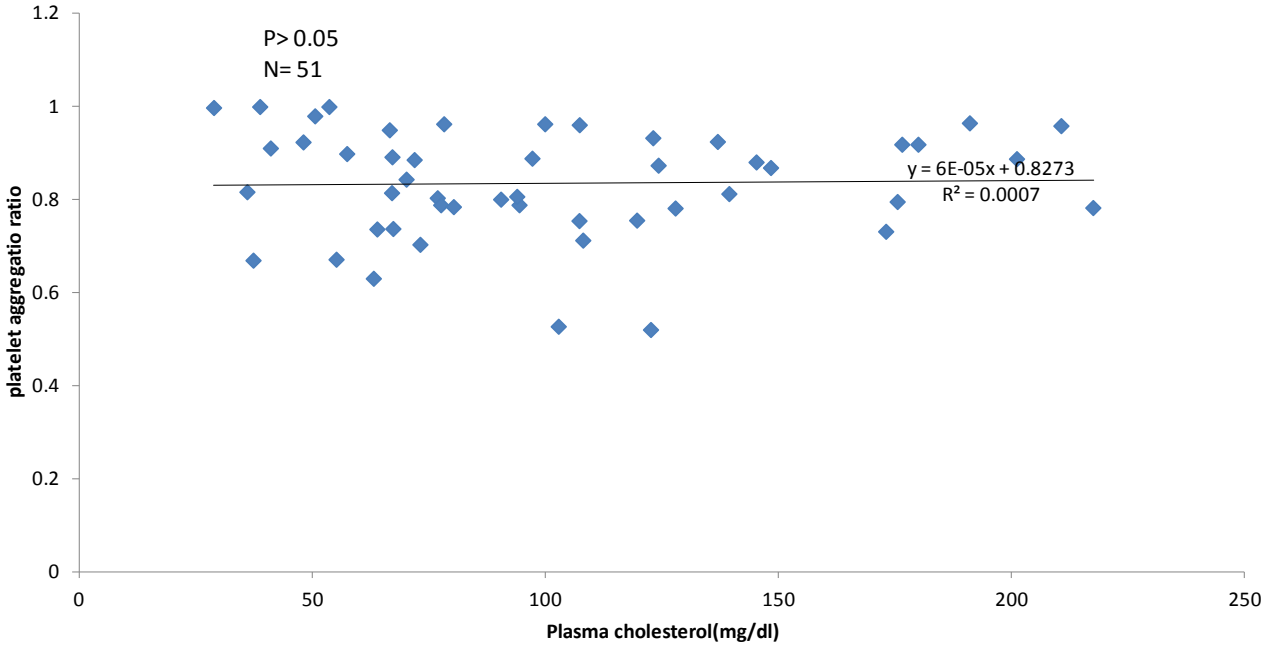

Figure 13. Scatter diagram showing correlation between platelet aggregation ration and plasma cholesterol in the control non diabetic subject.

not clear. But it may be as a result of several factors such as increased BMI, high blood glucose, triglycerides cholesterol levels.

In conclusion, this study in an African population suggests that, the increased platelet aggregation found in diabetic patients increased significantly with increases in BMI and with decrease Age. The study also suggests that, platelet aggregation in diabetic patients is associated

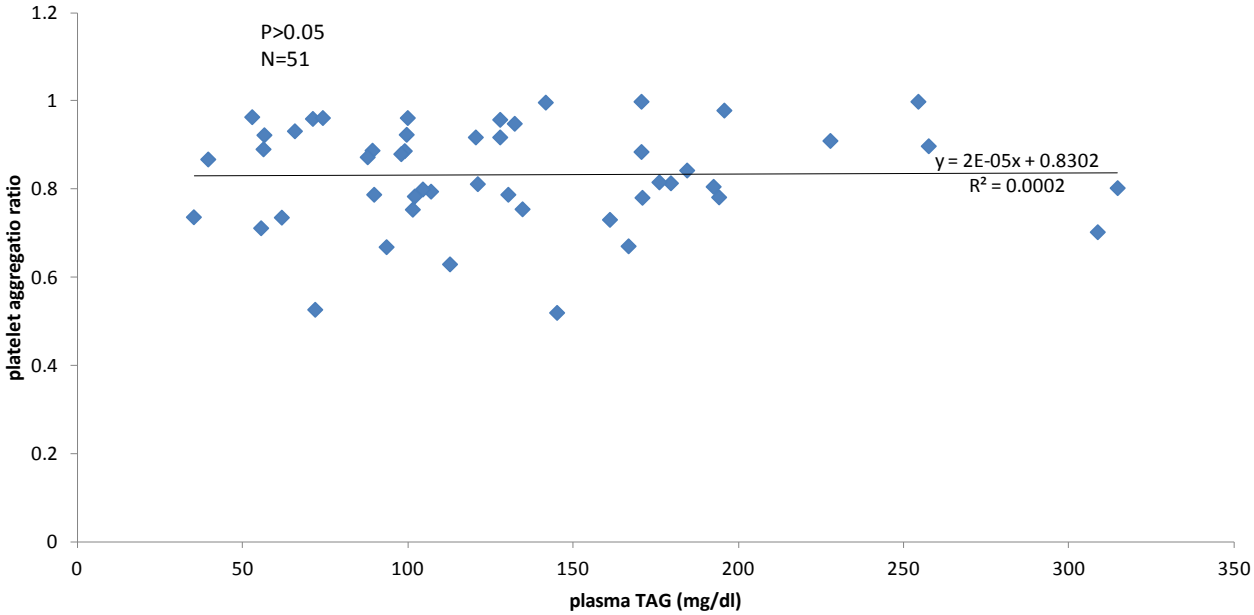

Figure 14. Scatter diagram showing correlation between platelet aggregation ration and TAG in the control non diabetic subject (Triglyceride).

with the increase metabolic imbalance in the diabetic patients. The limitation of this study is that it consider a small location in Nigeria with small sample size, therefore further study is require that consider diabetic patient from several location to establish the influence of metabolic imbalance and age on platelet aggregation in Nigeria diabetic patients.

Conflict of Interests

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

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African Journal of Biotechnology

Full Length Research Paper

Isolation, partial purification and characterization of antifungal trypsin inhibitor protease from the seed of *Blighia sapida* K. D. Koenig (*Ackee*)

Nwozo S. O.[1,2]*, Iwuoha E. I.[2], Waryo T.[2] and Kgarebe B.[2]

[1]Nutritional / Industrial Biochemistry unit, Department of Biochemistry, FBMS, College of Medicine, University of Ibadan, Ibadan, Oyo State, Nigeria.
[2]SensorLab, Department of Chemistry, University of Western Cape, Bellville, Cape Town 7535, South Africa.

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Seed proteins have been evaluated for their nutritive value and biological activity. Proteases and proteinase inhibitors have been of immense benefit to both agriculture and therapeutic purposes. The seed proteins of *Blighia sapida* was evaluated for both structure and biological activity in this study. The matured dried seeds were pulverized and sequentially extracted using 10 mM Tris/HCl pH 7.4, 10 mM ammonium acetate and 10 mM sulphuric acid. Crude protein extracts were concentrated and the protein concentrations were estimated. Proteins were purified by 70% ammonium sulphate precipitation, Sephadex G50 reversed phase chromatography and finally by HPLC on a C18 column. Two bands were obtained from SDS-PAGE electrophoresis and they were identified by ESI/MS using in gel tryptic digestion. The seed protein from *B. Sapida* consists of two single polypeptide chains each with mass of about 24 to 27 KDa as established by a combination of SDS-PAGE and ESI/MS. Proteins exhibited protease activity, which was confirmed by zymography. Protease activity was characterized for effect of temperature, pH, divalent metal ions and chelating agents. The crude protein from the seed of *B. sapida* showed antimicrobial activity and the antifungal activity was comparable with the reference drug, Ticonazole.

Key words: *Blighia sapida,* chromatography, protease activity, in-gel trypsin digestion/mass spectrophotometry, antimicrobial activity.

INTRODUCTION

Ackee, the national fruit of Jamaica, is a food staple in many Jamaican diets (Sharma et al., 2009). The ackee tree is a tropical evergreen tree that can grow as tall as 40 feet. Its leaves are broad and pinnate; it's approximately 10 cm wide, 100 g of the fruit may be colored anywhere from straw to bright red. *Blighia sapida* is a woody perennial multipurpose fruit tree species native to the Guinean forests of West Africa. The fruit

*Corresponding author. Email: sonwozo@yahoo.com, onyenibe.nwozo@mail.ui.edu.ng.

splits open while still on the tree to reveal 3glassy black seeds surrounded by a thick, oily, yellow aril. An association between ackee poisoning and Jamaican vomiting sickness was made in 1875 with a water-soluble toxic material in the seed and pods of the ackee fruit (Joskow et al., 2006; Moya, 2001; Gaillard et al., 2011). Hassal and Reyle (1954) were the first to isolate two toxic compounds in their crystalline form from the unripe fruit and the compounds were called hypoglycin A and B because of their hypoglycemic activity. The fruit should be allowed to open and ripen naturally on the tree as fresh arils of the ripened fruits are edible but the unripe fruit is not. *B. sapida* (Ackee) a known fish poison and the hypoglycin A and B have been identified as the toxicant in the seed (Bowen-Forbes and Minott, 2009; Moya, 2001; Natalini et al., 2000). The fruit is rich in essential fatty acids, vitamin A, zinc, and protein (Oladiji et al., 2009).

Commonly, seeds are evaluated for their suitability for nutritional and probable anti-feedant properties. Plants contain a variety of proteins which are resident in vacuoles and it includes storage proteins, hydrolases, proteases and α-amylase inhibitors as well as enzymes involved in plant defense (Hermann, 1994). Majority of proteinase inhibitors studied in plant kingdom originates from three main families namely leguminosae, solanaceae and gramineae (Richardson, 1991).

Leguminosae seeds contain high amounts of protein, a small portion of which consist the inhibitors that suppress proteolytic activity either *in vivo* or *in vitro*. Proteinase could be inhibited by various compounds including proteins by the formation of stable stoichiometric complexes, thus inhibiting their activity, and preventing proteolysis (Laskowsky and Kato, 1980). The presence of proteinase inhibitors in plants and seeds frequently accounts for the low nutritive value of uncooked vegetarian food (Liener, 1996). Studies have shown that human populations which are known to consume food with high concentration of proteinase inhibitors in their diet have lower rates of colon, breast, prostate and skin cancers. The protein proteinase inhibitors are divided into families according to the class of proteolytic enzymes inhibited, extensive sequential and structural homology among the members, the locations of disulfide bridges and the reactive site. The serine proteinases are the most widely studied (Macedo and Xavier-Filho, 1992; Macedo et al., 2002; Macedo et al., 2004). Various legume proteinase inhibitors have been classified as Kunitz-type, Bowman-Birk-type, potato I, potato II, squash, cereal super family, thaumatin-like and Ragi AI inhibitors (Richardson, 1991). Seed storage proteins often are not just protein stores for germination requirement and food for man alike but could provide a defense mechanism to protect the seeds from pathogen invasion. Many seeds such as *Adenanthera pavonina, Benincasa hispida, Areca catchu, Capparis spinosa* their seed proteins have been characterized as well as biological activity against common bean weevil and HIV-I reverse transcriptase activity (Macedo et al., 2004; Lam and Ng, 2008; Shih et al., 2001; Kusumoto et al., 2006). Preliminary study revealed potent antimicrobial and antifungal activity from the seeds and these considerations have led us to purify and characterize the seed proteins from *B. sapida*, which to the best of our knowledge has not been previously characterized.

MATERIALS AND METHODS

Chemicals

All chemicals used were either of analytical grade or the highest available purity. All solutions were prepared in MilliQ Water (Millipore, Bedford, MA, USA); EDTA (ethylenedinitrilo tetraacetic acid disodium salt) from Merck, Darmstadt, Germany; BIS (*N*, *N*-methylenebis-acrylamide), and acrylamide was obtained from Sigma (St. Louis, MO, USA); ammonium per sulfate (APS); bromophenol blue (3,3,5,5-tetrabromophenolsulfonephthalein); tetramethylethylene diamine (TEMED); 2-mercaptoethanol; glycerol, sodiumdodecyl sulphate (SDS), DL-dithiotreitol were purchased from Sigma as well. Crystallized and lyophilized trypsin, tris (hydroxymethyl) aminomethane hydrochloride (tris-HCl), ammonium bicarbonate, agar, divalent salts were from Sigma; all other reagents used were of the highest purity grade. Nutrient broth 'E' (Oxiod, England), Tryptone Soya Broth (Merck, Germany). Nutrient Agar, Sabouraud Dextose Agar and Sabouraud Dextrose Broth are all products of Becton Dickinson and company, U.S.A.

Microorganisms

Microorganisms used are *Staphylococcus aureus* (ATCC 13709), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (Clinical isolate), *Escherichia coli*, *Candida albicans* (ATCC 10231) and *Aspergilusniger.*

Seed collection and extraction

B. sapida seeds were harvested from the Botanical garden, University of Ibadan and were identified by Mr D Esimekhuai in the Herbarium of Botany Department, University of Ibadan, Ibadan, Nigeria. Seeds were air dried in Nutritional/Industrial Biochemistry laboratory in University of Ibadan and sorted to homogeneity. Air dried seeds were milled with coffee grinder and stored in air tight bags until required. Fifty grams of the matured dried seeds of *B sapida* were pulverized and was sequentially extracted by cold maceration for 24 h with 10 mM Tris /HCl pH 7.4, 10 mM ammonium acetate and 10mM sulphuric acid.

Protein concentration determination

The protein concentration was measured spectrophotometrically at 280 nm for the 3 fractions obtained from chromatographic separations or total protein concentration in the eluted fractions as well as the purified protein was determined using the method of Bradford 1976 with Bovine serum albumin (BSA) as standard.

Purification and fractionation of crude proteins

Crude proteins from the extractions were concentrated on Bucchi

Rotavap 114 at 30°C to reduce volumes to about 30ml. This was followed by ammonium sulphate precipitation (70%) and precipitates were separated by centrifugation at 10,000 rpm for 20 min at 4°C. Residues were dissolved using a few mills of 10mM ammonium acetate buffer containing 0.001% sodium azide and stored at -20°C. Crude fractions from ammonium sulphate precipitation was separated on Sephadex G50 on a Flex column 2.5 by 100 cm, the column using 10mM ammonium acetate buffer containing 0.001% sodium azide as running buffer. Flow rate was maintained at 1 ml/8min using a fraction collector and the eluent was monitored at 280 nm spectrophotometrically on GBC UV/V is 920 instruments to obtain chromatogram. Chromatogram from size exclusion sephadex G50 separation was used to obtain the different fractions; peaks 1, 2 and 3 from the three different extractions from *B. sapida* seeds and these were pooled together and concentrated using rotary evaporator.

Final purification of the seed protein was achieved by subjecting the protease active fraction from sephadex G50 separation to HPLC using C18 (5 µm, 46 × 250 mm; Vydac, the Separation Group, Inc., USA) Zymographic determination reversed phase column. The elution was performed under the following conditions: eluent A, 0.1% TFA in water (v/v), eluent B 100% acetonitrile and 0.05% TFA. Gradient program was 5% B for5 min, 0 to 60% B in 20 min followed by 100% B for 25 min on Agilent technologies 1200 series instrument. The flow rate was maintained at 1 ml/min and absorbance was monitored at 230 nm. Aliquots were taken from each purification stage for both Bradford protein concentration estimation and SDS/PAGE electrophoresis.

SDS polyacrylamide gel electrophoresis

The purity and molecular mass of the different fractions of partially purified enzyme and crude seed protein was established by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The separating gel (10%) was prepared by mixing 2.0 mL deionized water, 1.25 mL Tris-HCl (buffer 1.5 mol/L, pH 8.8), 50 μL SDS (10% (w/v)), 1.66 mL acrylamide (30% (w/v))/bismethylenacrylamide (0.8% (w/v)), 25 μL APS (10% (w/v)), and 15 μL TEMED. This gel was polymerized for 45-60 min at room temperature. Next the stacking gel (4%) was prepared by mixing 1.195 mL water, 0.5 mL Tris-HCl (0.5 mol/L, pH 6.8), 20 μL SDS (10% (w/v)), 0.26 mL acrylamide (30%) and bis-methylenacrylamide (0.8%), 15 μL APS (10% (w/v)), and 10 μL TEMED. These gels were polymerized within 35-45 min. After 5 μL of pure protein samples and 15 µL of impure protein extracts were heated at 100°C for 5 min and applied electrophoresis was run at 150 V over the gel. Finally the gel was stained by a solution of Coomassie brilliant blue (0.25% (w/v)) and destained in (10% acetic acid, 30% methanol and 60% deionized water (v/v) destaining solution overnight and gels in gel storage packs were scanned using 3 in 1 (2050 Hewlett-Packard printer).

Zymography

of the protease activity was done as above except for the incorporation of 1% gelatine in the gel as described by Lacks and Springhorn (1980) and samples were only dissolved in sample diluting buffer without heating. Briefly after electrophoresis, the gel was washed with 2.5% Triton-X 100 (3 × 20 min) with continual shaking to remove SDS followed by washing with water and incubation for 48 h at 37°C in activation buffer (25 mM Tris-HCl buffer added 5 mM $CaCl_2$ of pH 7.4). Finally, the gel was stained with coomassie gel staining dye and destained with 10% acetic acid and 30% methanol solution to visualize the clear bands of proteolysis against the dark background.

Protease agar plate /trypsin inhibitory assay

Method of Pfleinderer and Krauss (1965); Scumacher and Schill 1972 were used. Briefly, 0.5g of casein was dissolved in 25 mL of 50 mM Tris-HCL buffer pH 8.0 to which 1 mM calcium chloride and 0.01% sodium azide has been added with gentle stirring for 1 h, solution was made up to 50 ml with the pH adjusted to 8.0. 1 g of agar was heated in 25 ml of casein buffer in the microwave for about 50 s, it was cooled to about 50-60°C and mixed with casein solution (1:1) and poured into petri dishes and degassed to solidify in a Laminar Flow hood. Wells were formed using sterile cork borer.

Protein extracts at different concentrations were added to the wells and incubated at 37°C for 24 h for agar plate protease assay while trypsin and plant extracts was added to the wells for trypsin inhibitory assay. At the end of incubation agar plates are exposed to saturated ammonium sulphate (1M) and zone of white precipitate indicates catalytic activity. In the trypsin inhibitory assay difference in diameter between well of trypsin alone and that with plant extract was used to calculate inhibition from Trypsin activity unit (au) of 1645 in 1ml/mg.

Enzyme activity assay

Spectrophotometric protease activity of crude protein extract and partially purified protein fraction was done by slight modification of Kunitz and McDonald (1946). Briefly, 20 µg of crude protein, 100 µL 1% (w/v) Casein was dissolved in 0.1 M tris –HCl pH 8.0 buffer. The reaction mixture was incubated at 37°C for 30 min in Gefran 500, temp control unit and the reaction was stopped by adding 200 µL of 40% Trichloroacetic acid (TCA). Blank was prepared for each sample in a similar manner except that the 20 µg of crude protein was added after reactionhas been quenched with 40% TCA.The samples were centrifuged at 14,000 rpm in Eppendorf mini spin plus for 10 min and absorbance of TCA soluble peptides was measured at 280 nm. Activity unit was calculated using the difference in absorbance between sample and blank, and all measurement were done in triplicate and average values were used.

Effect of temperature and pH on protease activity

The effect of temperature on protease activity was determined by incubating the enzyme reaction mixture at different temperatures ranging from 37 to 90°C. Reaction mixture was 20 µg of respective crude protein, 100 µL 1% (w/v) Casein 0.1 M tris –HCl pH 8.0 buffer and was at 37, 45, 50, 60, 70, 80 and 90°C for 30 min in Gefran 500, temp control unit. The reaction was stopped by addition of 200 µL of 40% Trichloroacetic acid (TCA). Blanks were similarly prepared for each sample, incubated at the different temperatures, except that the 20 µg of crude protein was added after reaction has been quenched with 40% TCA. Samples were centrifuged at 14,000 rpm in Eppendorf mini spin plus for 10 min and absorbance of TCA soluble peptides was measured at 280 nm. Protease activity unit at the various temperatures were calculated using the difference in absorbance between sample and blank. Each measurement was done in triplicate and average values were used to obtain protease activity at the different temperatures.

The effect of pH on protease activity was determined by testing for protease enzyme activity using standard laboratory method earlier described except that 100 µL of 1% (w/v) Casein was prepared in 0.1 M buffers of different pH values.The buffers used were 50 mM Glycine-HCl buffer (pH 3.0), sodium acetate buffer (pH 4.0 to 5.0), sodium phosphate buffer (pH 6.0 to7.0), Tris-HCl buffer (pH 8.0 to 9.0) glycine –sodium hydroxide buffer (pH 10.0 to 11.0). Triplicates of reaction mixtures and the corresponding blanks (without) crude proteins were incubated for 30 min at 37°C and the assay was

continued as earlier described for protease activity determination.

Effect of metal ions and chemical agents

The protease activity was then measured as earlier described and percentage inhibition was calculated by comparison with optimum conditions. Divalent metal ions such as Sn, Mg, Pb, Fe, Mn, Cu and Co were used as the chloride salt to determine effect of metal ions. Metal chelator such as EDTA, sulphydryl agent ditthiothreitol and detergents such as triton-x, sodium dodecyl sulphate and urea were used for effect chemical agents. The effect of metal ions and chemical agents on protease activity was determined by incubating the enzyme with 1 mM of the respective chelating agent or metal ion for 30 min at 50°C. Briefly, assay mixture was prepared using 20 µg of respective crude protein, 100 µl 1% (w/v) Casein 0.1 M tris –HCl pH 8.0 buffer, 1 mM of the metal ion or chemical agent and incubating at 37°C for 30 min in Gefran 500, temp control unit. Reaction blank was similarly prepared except that the enzyme was added after reaction had been stopped using TCA and reactions were continued as earlier described for spectrophotometric protease activity measurement.

HPLC purification

Final purification of the seed protein was achieved by subjecting the protease active fraction on C18 (5 µm, 46 × 250 mm; Vydac, the Separation Group, Inc., USA) reversed phase column on Agilent technologies 1200 series instrument. The elution was performed under the following conditions:

Eluent A, 0.1% TFA in water (v/v), eluent B 100% acetonitrile and 0.05% TFA. Gradient program was 10% B for5 min, 0 to 100% B for 15 min.

The flow rate was maintained at 1 ml/min and absorbance was monitored at 254 nm. Representative HPLC chromatogram for BS Tris first peak is shown in Figure 3a, all *B sapida* extracts from Tris, Amac and Sulp had similar chromatographic profile except the observed decrease in the peak at 8 to 10 min possibly due to decreased concentration from subsequent extraction from the same starting see powder

In gel trypsin digestion / mass spectrometry

All gel pieces were cut into smaller cubes, the gel pieces were digested with 20 µL of a 10 ng/µL trypsin solution at 37°C overnight. The resulting peptides were extracted twice with 70% acetonitrile in 0.1% formic acid for 30 min, and then dried and stored at -20°C. Dried peptides were dissolved in 5% acetonitrile in 0.1% formic acid and 10 µL injections were made for nano-LC chromatography.

All experiments were performed on a Thermo Scientific EASY-nLC II connected to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electropsray source. For liquid chromatography, separation was performed on an EASY-Column (2 cm, ID 100 µM, 5 µM, C18) pre-column followed by an EASY-column (10 cm, ID 75 µM, 3 µM, C18) column with a flow rate of 300 ml/min. The gradient used was from 5 to 40% B in 20 min, 40 to 80% B in 5 min and kept at 80% B for 10 min. Solvent A was 100% water in 0.1% formic acid, and solvent B was 100% acetonitrile in 0.1% formic acid. The mass spectrometer was operated in data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Data were acquired using the Xcalibur software package. The precursor ion scan MS spectra (*m/z* 400 – 2000) were acquired in the Orbitrap with resolution R = 60000 with the number of accumulated ions being 1 × 106. Data analysis was done using Thermo Proteome Discoverer 1.3 (Thermo Scientific, Bremen, Germany) were used to identify proteins via automated database searching (Mascot, Matrix Science, London, UK) of all tandem mass spectra against the Swissprot 57.15 databaseof more than $p<0.05$ and peptides of high and medium confidence as determined by Proteome Discoverer.

Antimicrobial assays

The agar diffusion method was used (Reeves et al., 1978). 0.1ml of 1 in 100 dilution of the overnight broth culture of each organism was used to seed sterile molten nutrient agar maintained at 45°C. The plates were allowed to solidify and six wells (8 mm diameters) were made in the seeded plates. Different dilutions of the crude *B. sapida* seed protein ethanol extract (400, 200, 100, 50 mg/ml) were added into the appropriate wells. The plates were incubated at 37°C for 24 h; Ampicillin (10µg/ml) was used as positive control and dimethylsulphoxide (DMSO) was used as negative control. Antifungal tests were performed in a similar manner seeded with fungi hyphae in sabouraud dextrose agar petri- dishes. A suspension of the organism (fungi) was prepared by inoculating a small amount into Typtone Soya Broth (TSB). Tioconazole ('Trosyd' 5mg/ml) was used as reference antifungal drug. All plates were subsequently incubated at room temperature for 48 h (Reeves et al., 1978; Cowan, 1974). The diameter of zone of inhibition was measured as an indication of activity.

RESULTS

Fifty gram *B. sapida* on extraction with 500 ml each of buffer, concentration and ammonium sulphate precipitation yielded the following:

10 mM Tris buffer PH 7.4 total volume 32 ml (3.027 mg/ml); 10 mM Ammonium acetate total volume 10 ms^{-1} (0.998 mg/ml) and 10 mM Sulphuric acid gave total volume 10 ml (2.046 mg/ml).

Impure protein from the three extracts from 50 g BS seed was 127.304 mg (0.2546%) percentage yield. Obtained chromatograms from size exclusion chromatography are shown as (Figures Ia, b and c) while the protein concentrations of the concentrated pulled chromatographic fractions are shown on Table 1. Separation with the sephadex G50 size exclusion yielded 60.181mg (47.27%) and about 2 fold purification. Similar chromatogram was obtained from OD_{280} measurements for BS Tris, BS amac and BS sulpsize exclusion chromatography using sephadex G50 as shown (Figures Ia,b and c) from which we obtained 3 peaks each. Preliminary screening using the agar plate assay for protease activity showed test tube 32 had highest protease activity, followed by 30 and 25, whiletest tube 51 showed minimal activity andtest-tubes 95, 92, 81 and 61 showed no activity.

SDS-PAGE electrophoresis bands from the crude *B. sapida* extract was rather broad but better bands were obtained after size exclusion chromatography and desalting (Figure 2a). All three peaks of *B. sapida* from

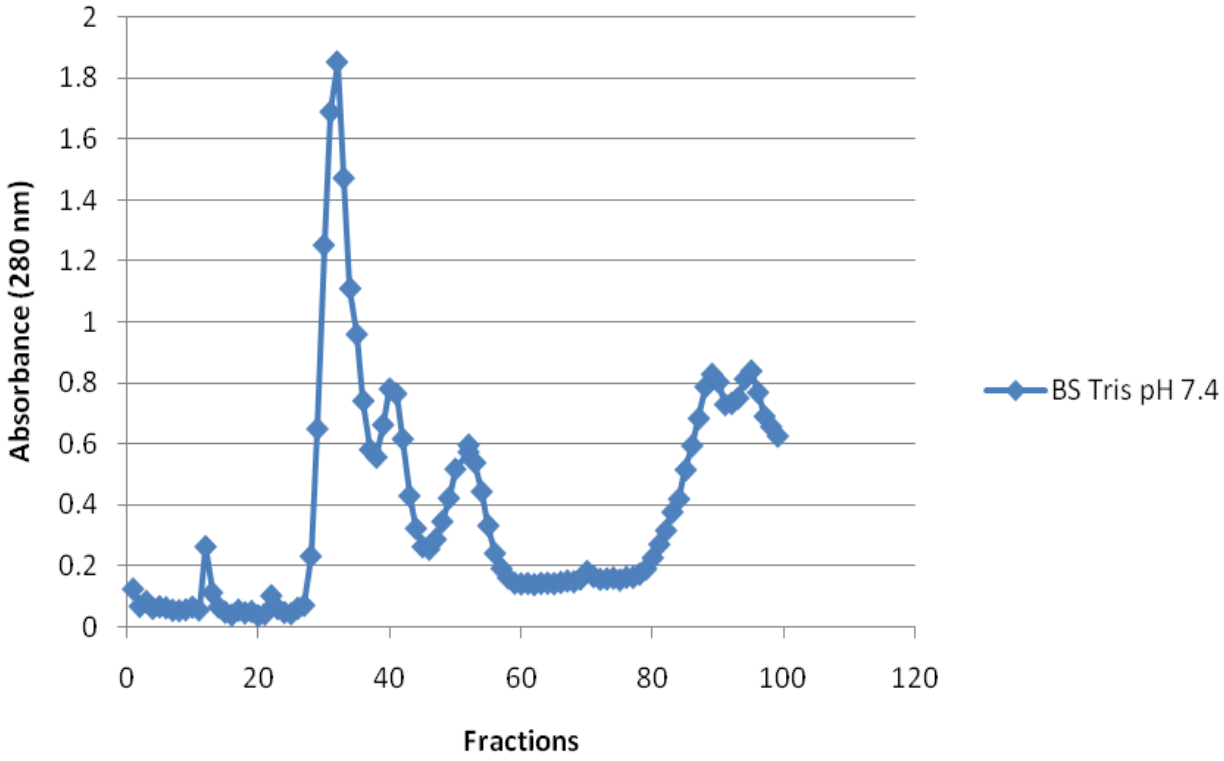

Figure Ia. Chromatogram for the sephadex G50 separation of *B. sapida* 10 mMTris /HCl pH 7.4 extract on Flex column 2.5 by 100 cm and flow rate of 1 mL/8min monitored at 280nm.

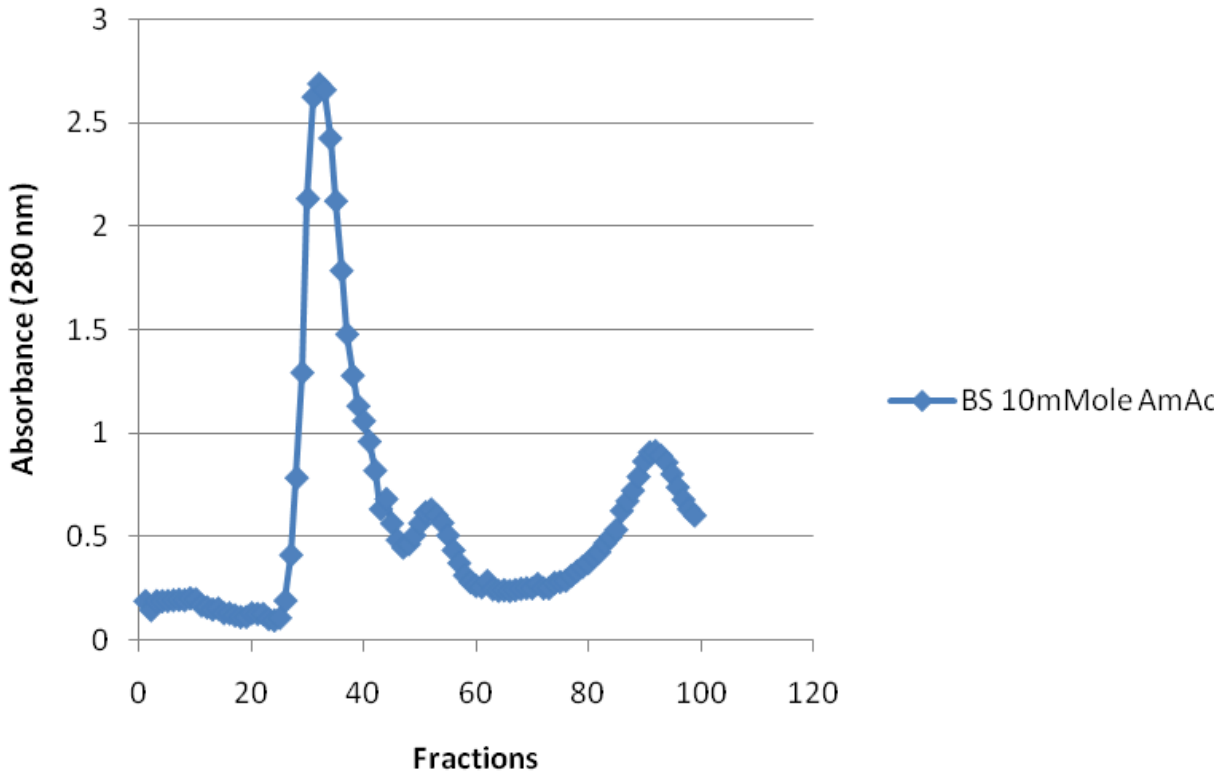

Figure Ib. Chromatogram for the sephadex G50 separation of *B. sapida*10 mM ammonium acetate extract on Flex column 2.5 by 100 cm and flow rate of 1 mL/8min monitored at 280 nm.

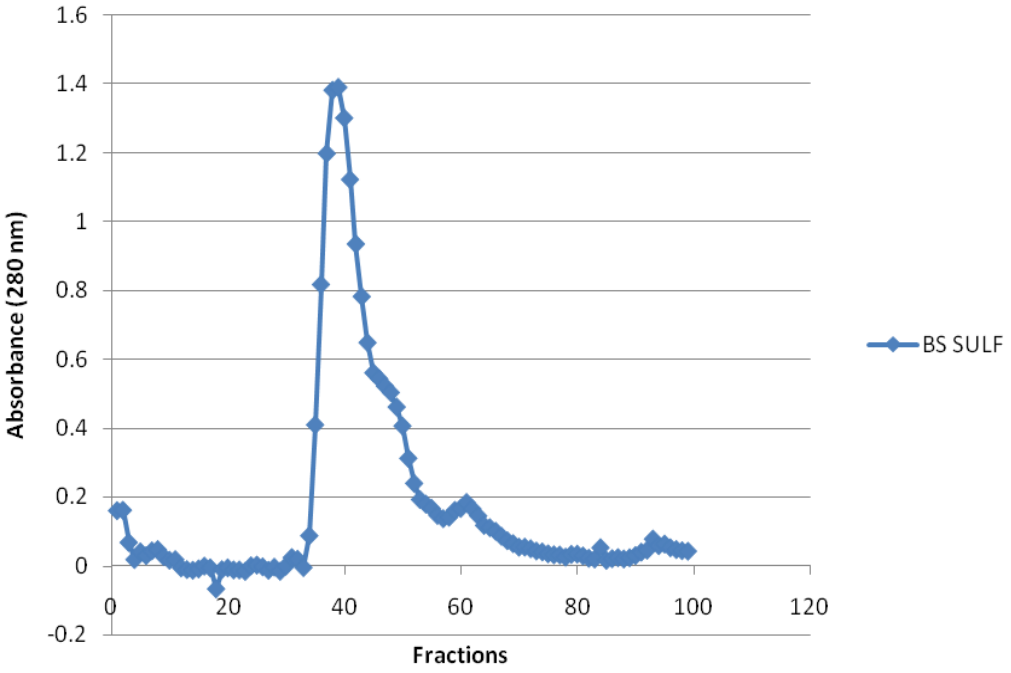

Figure Ic. Chromatogram for the sephadex G50 separation of *B. sapida* 10mM sulphuric acid extract on Flex column 2.5 by 100 cm and flow rate of 1 mL/8min monitored at 280 nm.

Table 1. Protein concentration estimations of size exclusion chromatography fractions using sephadex G50.

Peak	Amount of protein (mg)		
	10 mM Tris	10 mM Amac	10 mM sulp
1st	20.406	2.40	5.49
2nd	15.625	1.60	3.75
3rd	7.48	1.75	1.68
Total protein	43.511	5.75	10.92

sephadex G50 on SDS/PAGE gave two similar bands (Figure 2b) of about 20 to 30 KDA using marker proteins. The purity and molecular mass of the different fractions as well as purified enzyme was established by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Likewise, zymographic determination of the protease activity was established by light patch in the dark background of the 1% gelatingel as shown in Figure 2c. The nine chromatographic fractions (ie peaks 1, 2 and 3) from Tris, Amac and Sulp extracts from sephadex G50 (Figures Ia-c) where further purified using HPLC and we observed similarprofile for the fractions except decrease in the peak height of eluent at 9-10 and 14.7-15.3 min, which may possibly be due to decreased concentration from subsequent extraction from the same starting seed powder and purification processes. Representative HPLC chromatogram for BSTris first peak and BSsulp 3rd peak are shown in Figures 3a and b. In-gel mass spectrophotometry of the two bands from SDS/PAGE electrophoresis is shown on Figures 4a and b respectively.

Trypsin protease inhibitory agar plate assayed shows that the partially purified protease inhibited trypsin activity in a concentration dependent manner, and protease activity of extract only decreased from BS Tris> BS Amac> BS sulpas shown on Table 2 from diameter measurement of zone of inhibition. BS Tris extract was virtually inhibited by all divalent metals used in this study except Cu^{2+} with on 6.67% activity while BS Amac and BS Sulp had varying activity except with Co ions where protease activity was totally absent (Table 3a). Metal chelator, sulphydryl reagents and detergents had varying effect on protease activity (Table 3b). Optimum temperature and pH for protease activity were found to be 50°C and pH 5.0 respectively for BS Tris extract (Tables 3c and d). *B. sapida* crude extract showed

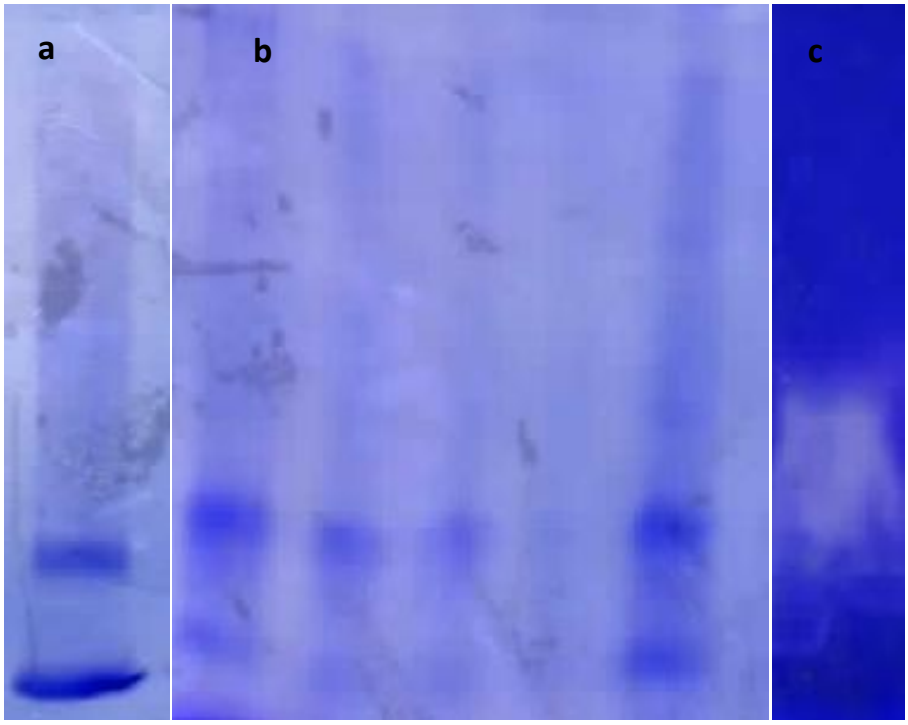

Figure 2. a) Examination of crude protein from the Sephadex G-50 run; b) SDS –PAGE electrophoresis showing Tris 1st, 2nd, 3rd peaks and Amac 1st and 2nd peaks from Sehpadex G50 separation; c) SDS-PAGE zymography of 1st peak of Tris extract with light patch showing zone of protease activity.

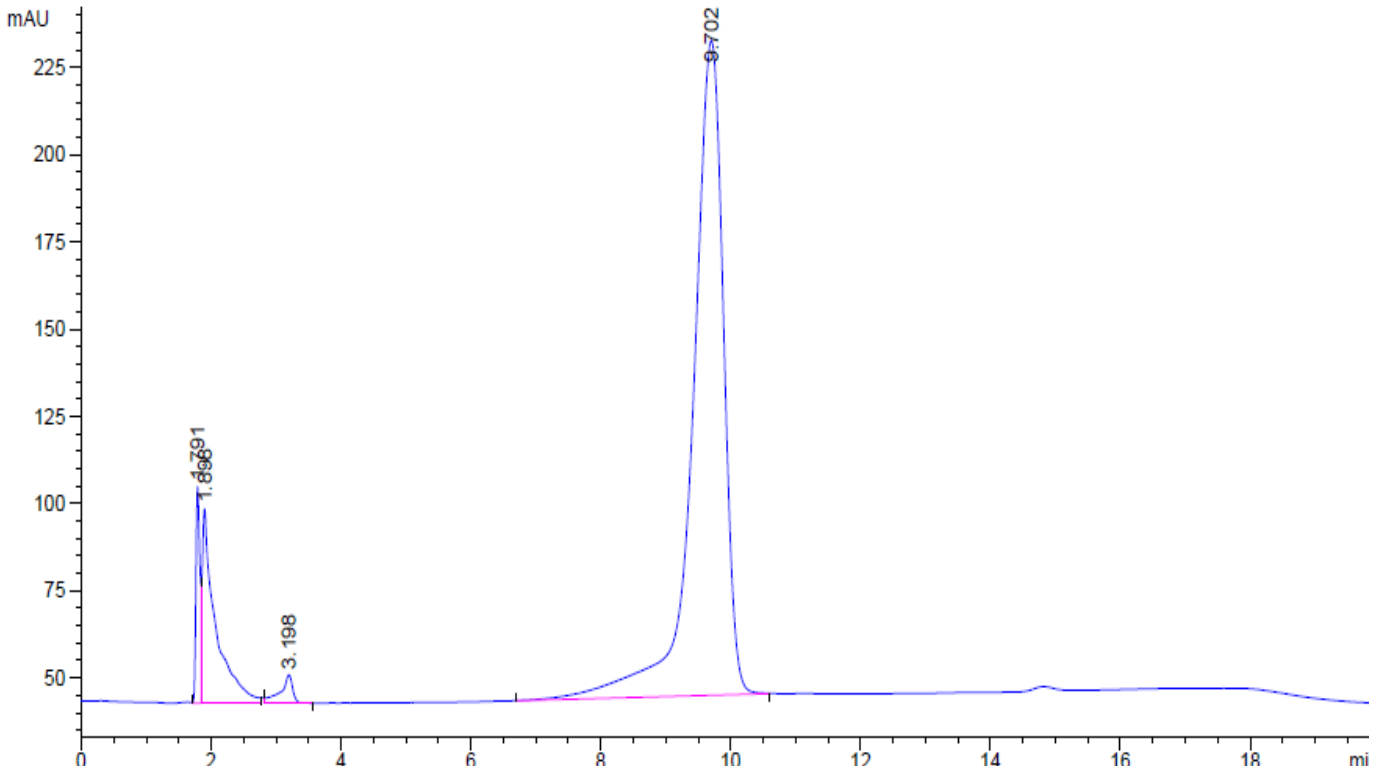

Figure 3a. HPLC chromatogram for the separation of *B.Sapida* 10 mMTris/HCllst peak fraction obtained from sephadex G50 size exclusion chromatography.

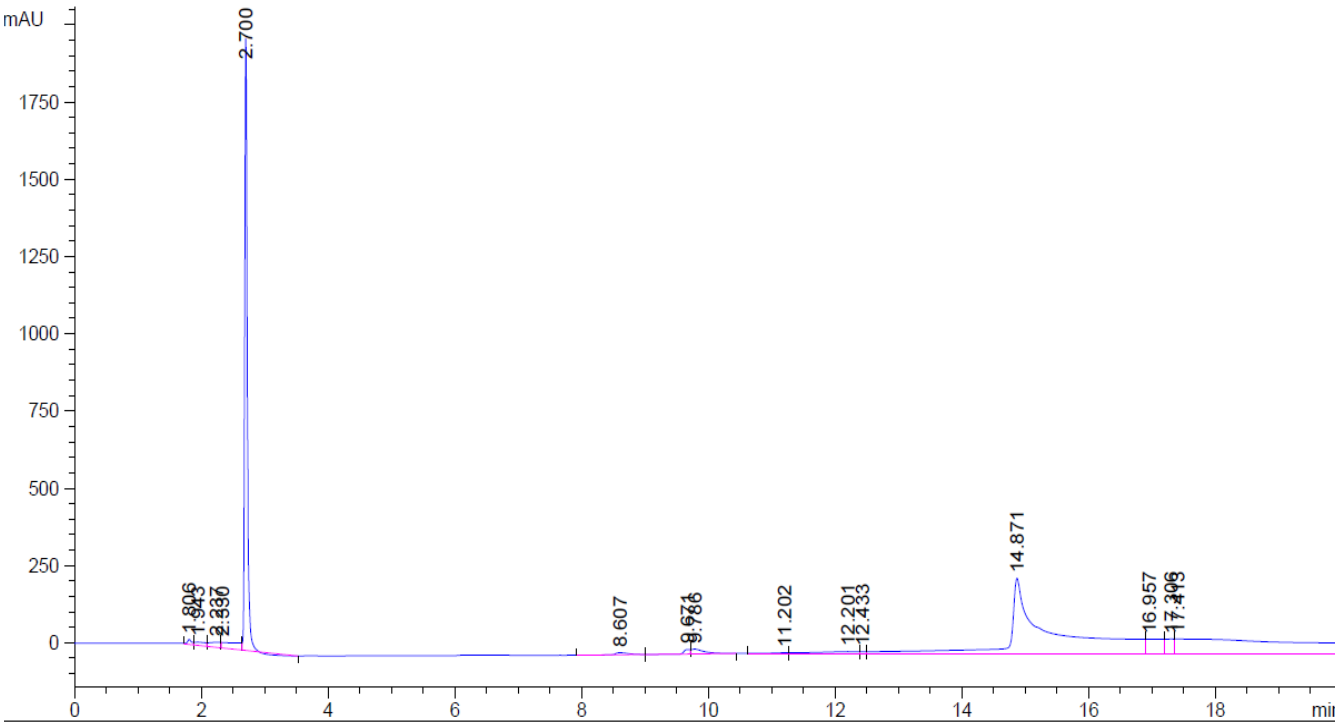

Figure 3b. HPLC chromatogram for the separation of B. *Sapida* 10 mMsulphuric acid 3rd peak fraction obtained from sephadex G50 size exclusion chromatography.

Table 2. Trypsin agar plate inhibitory assay of the crude protein extract of Blighia sapida.

Well content	Diameter zone of inhibition (cm)	Activity unit(au) calculated from % inhibition	
		10µg	20µg
Trypsin only	0.8 0	64.63	78.57
BsTris + Tryps	0.55	54.05	65.71
BsAmac + Trysin	0.46	37.60	45.71
BsSulp + trypsin	0.32		
BsTris (20µg)	0.60		
BsAmac (20µg)	0.48		
BsSulp (20µg)	0.35		

Diameters of zone of inhibition was measured from edge of well to periphery of white casein precipitated zone on the addition of saturated ammonium sulphate solution.

Table 3a. Effect of different divalent metal ions onspectrophotometric protease reactivity at 37°C.

Metal ion	Relative percentage protease activity (%)		
	BsSulp	BsTris	BsAmac
Sn	103.3	ND	82
Pb	80.83	ND	25
Mg	ND	ND	76.6
Fe	ND	ND	100
Cu	116.67	6.67	100.56
Co	ND	ND	ND
Mn	ND	ND	15.5

Table 3b. Effect of chemicals agents and chelators on protease activity (%) of *B. sapida* extracts from sephadex G50 separation.

Agent	T^1	T^2	T^3	A^1	A^2	A^3	S^1	S^2	S^3
EDTA	0	24.67	66.67	63.3	55.6	70.3	68.3	0	0
Urea	0	0	0	0	0	0	66.6	51.6	0
Dithioerythrol	83.3	0	0	0	82.2	0	0	0	0
Triton – X	0	0	0	0	4.84	0	4.8	0	30
SDS	0	0	34.6	76.7	75.5	51.1	61.6	0	50

Table 3c. Effect of temperature on*B.sapida* 10mM Tris/HCl sephadex G50 separated seed protease activity

Temperature in °C	Relative activity
37	0.018
45	0.02
50	0.026
60	0.015
70	0.014
80	0.005
90	Nd

Table 3d. Effect of pH on *B. sapida* 10 mM Tris /HCl sephadex G50 separated extract seed protease activity

pH	Protease activity
3	0.005
4	0.0165
5	0.0275
6	0.017
7	0.015
8	0.018
9	0.014
10	0.008
11	0.005

minimal antibacterial activity in the agar plate dilution assay which was concentration dependent (Table 4a) but the antifungal activity of the seed protein was comparable with the reference drug (Table 4b).

DISCUSSION

B. sapida seed proteins exhibited proteolytic activity in both agar plate assay, protease activity assay (spectrophotometric method) as well as exhibiting concentration dependent inhibition of trypsin. Protease activity for all nine fractions decreased slightly from 37 to 45°C before increasing from 50 to 80°C, above this temperature there was loss of activity (Table 3c). *B. sapida* exhibited protease activity over pH range 4.0 to 10.0 and above this pH there was loss of activity possibly due to denaturation (Table 3d). There was variation from decreased protease activity to non-detectable protease activity in the spectrophotometric assay using divalent metal ions such as Sn, Pb, Mg, Fe, Cu, Co and Mn by extracts of *B sapida* from Tris, Amac and Sulphuric acid fractions (Table 3a). However there was a slight increase in protease activity in the presence Cu ions BS amac (100.56%), BSsulp (116.67%) and Sn ions BSsulp (103.3%). Metal chelators such EDTA, sulphydryl reagent dithioerytrol and detergents such as Triton-X, SDS and Urea had varying effects on protease activity of BS fractions from Sephadex G50 separation (Table 3b) from inhibition to non-detectable activity.

All serine inhibitor families from plants are competitive inhibitors and all of them inhibit proteinases with a similar standard mechanism (Laskowki and Kato, 1980). *B sapida* proteases show a pH maximum at 5 to 7 and were very stable having thermophilic temperature range of protease activity between 50 to 70°C as shown on Tables 3c and 3d. The optimum activity of cysteine proteinases is usually in the pH range of 5 -7, which is the pH range of the insect gut (Murdoc et al., 1987).Serine proteinases have been described inmany plant species, and are universal throughout the plantkingdom, with trypsin inhibitors being the most common type(Lawrence and Koundal, 2002) possibly due to thefact that (mammalian) trypsin is readily available and is theeasiest of all the proteinases to assay using syntheticsubstrates, and hence it is used in screening procedures. On Table 2, the crude protein isolated exhibited but protease activity and trypsin inhibitory activity too. Such studies have provided a basic understanding of the mechanism of action (Huber and Carrel, 1989) that applies to most serine proteinase inhibitor families and probably to the cysteine and aspartyl proteinase inhibitor families as well.

Two analytical techniques are primarily employed in current proteomic research: two-dimensional (2D) gel electrophoresisforthe separation and visualization of proteins in crude extracts, and mass spectrometry (O'Farrel, 1975; Fenn et al., 1989) for the identification and characterizationof the separated proteins. SDS-PAGE electrophoresis, the proteins are separatedaccording to their molecular weights and a large numbers of proteinscan be separated and characterized by automated matrix-assisted laser desorption/ionization time-of-flight mass spectrometric

Table 4a. Antibacterial activity of the crude seed protein of *Blighia sapida.*

Microorganism	*B. sapida* (mg/ml)				Reference compound (mg/ml)- Gentamicin
	A	B	C	D	
Staphylococcus aureus	10	12	12	14	34
Bacillus subtilis	10	12	14	16	34
Pseudomonas aeruginosa	-	-	-	10	34
Escherichia coli	10	10	12	14	36

Gentamicin (25 µg/ml), A = 0.1250 mg/ml, B = 0.25 mg/ml, C = 0.5 mg/ml, D = 1 mg/ml corresponding to 12.5%, 25%, 50% and 100% dilutions respectively.Diameter of zone of inhibition (mm) 10 – 15 = +; 16 – 20 = + +; >20 = + + + ; 0 – 8 = no inhibition .Diameter of cup = 8mm

Table 4b. Antifungal activity of the crude protein extracts of *Blighia sapida.*

Microorganisms	*B. sapida* (mg/ml)				Tioconazole (Reference drug)
	A	B	C	D	
Aspergillus niger	-	10	12	14	20
Candida albicans	-	12	14	16	20

Tioconazole (5 mg/ml) A =0.125 mg/ml, B = 0.250 mg/ml, C = 0.5 mg/ml, D =1 mg/ml.
Diameter of zone of inhibition (mm): 10 – 15 = +; 16 – 20 = + +; >20 = + + +; 0 – 8 = no inhibition, Diameter of cup = 8mm.

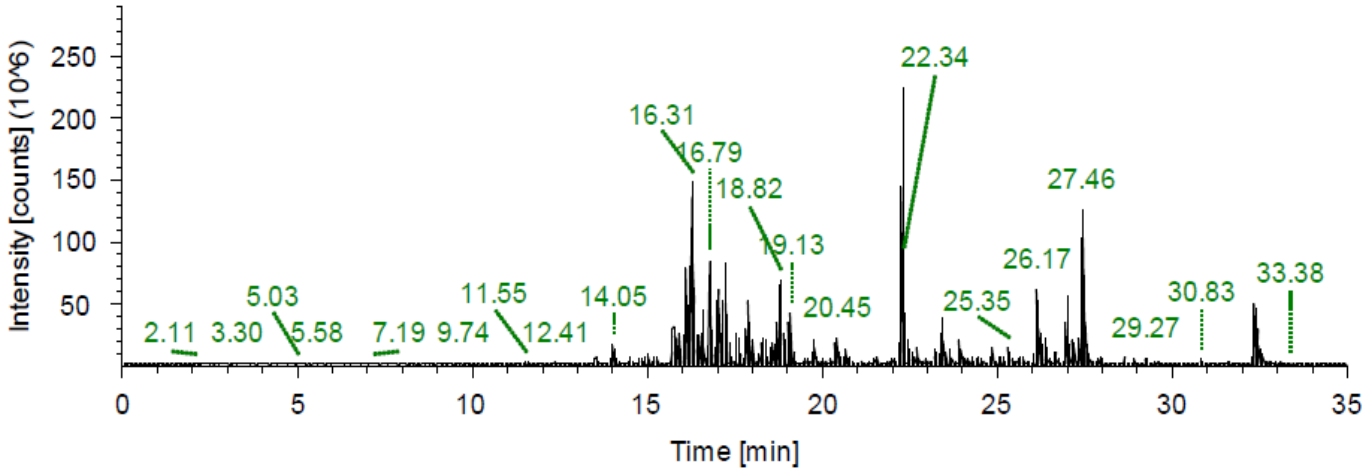

Figure 4a. In-gel trypsin digestion of the lower band of *B. sapida* was identified as Thaumatin like protein I peptide with 225 amino acid residues, 24.2KDA and pI of7.9

(MALDI TOF-MS) peptide mapping followed by extensive database searches (Henzel et al., 1993). In cases, wheremore structural information is required from the separated proteins, nano-liquid chromatography (LC)-electrospray ionization (ESI)-MS/MS is often employed (O'Farell 1975). Thedominant approach to proteomics includes theseparationof native proteins by 2-DE, their analysis bymass spectrometry (MS), and their identification basedon sequence information in databases.). Protein arrays provide a suitable sourceof a large number of recombinant proteins andthe storedMS information may be used to identify native proteinsfrom gels. The two bands from SDS/PAGE of *B. sapid*a crude seed extract were identified as Acidic endochitinase with 254 amino acid residues, mass of 27.6KDa, calculated isoelectric point of 6.02 and Thaumatin-like protein with 225 amino acid residues, mass of 24.2 KDa and a calculated isoelectric point (pI) of 7.9 (Figures 4a and b). This is in agreement with our data on effect of pH on protease activity especially as we only carried out partial

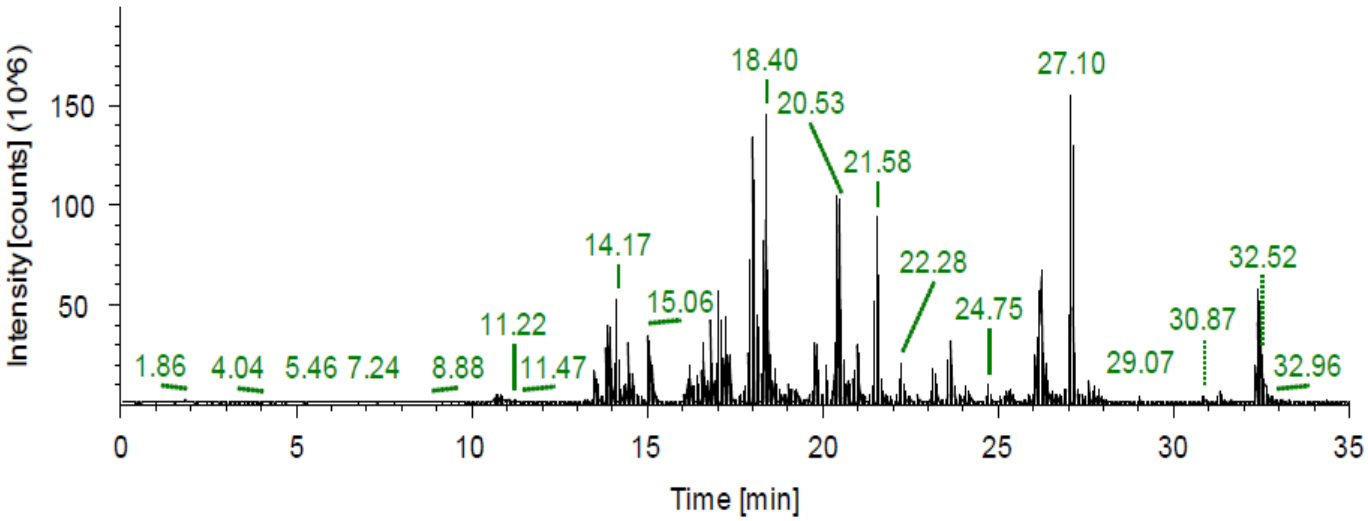

Figure 4b. In-gel trypsin digestion of the upper band of *B sapida* were identified as Acidic endochitinase with single peptide chain of 254 amino acid residues, KDA of 27.6, isoelectric point (pI) of 6.02.

separation. It might be necessary in future to collect the fractions from HPLC separations and identify the seed proteins individually. We observed antimicrobial activity in the crude seed protein, while the antifungal activity was approximately that of the reference drug Table 4b. This is not surprising as most seed proteins are not just present as seed storage proteins but protect the seed from pest and pathogens invasion.

In conclusion, we report the presence of two seed serine proteinase acidic endochitinase and Thaumatin-like proteins with mass of 27.2 and 24.2 KDa respectively in the seeds of toxic plant with promising antifungal activity. The seed is largely left to waste probably due to poisoning by hypoglycin A and B isolated from it but the protease and antifungal activity of the seed proteins might make for appealing industrial/technological application, especial as it retains proteolytic activity at high temperatures and over a wide pH range

Conflict of Interests

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

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