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Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)
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Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. Afr. J. Biotechnol. 7: 3535-3539.

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

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

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

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Review

Loop-mediated isothermal amplification (LAMP) based detection of bacteria: A Review

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Various diseases are caused by pathogenic bacteria and their diagnosis depends on accurate detection of pathogen from clinical samples. Several molecular methods have been developed including PCR, Real Time PCR or multiplex PCR which detects the pathogen accurately. However, every method has some limitations like low detection limit, whereas Loop-mediated isothermal amplification (LAMP) is a powerful and novel nucleic acid amplification method, which detects the DNA at very low level compared to other methods. This method amplifies very few copies of target DNA with high specificity, efficiency and rapidity under isothermal conditions by using a set of four specially designed primers and a DNA polymerase with strand displacement activity. This review presents detection of various bacteria by LAMP method and covers their detection limit in clinical specimens.

Key words: Bacteria, Loop-mediated isothermal amplification (LAMP), sensitive, rapid, simple.

INTRODUCTION

Isolation and characterization of pathogens from clinical samples is a tedious job. Traditional methods of microbial identification rely on the phenotypic characteristics like bacterial fermentation, fungal conidiogenesis, parasitic morphology, and viral cytopathic effects which are commonly used. Some phenotypic characteristics are sensitive enough for strain characterization; these include biotyping, isoenzyme profiles, antibiotic susceptibility profiles, and chromatographic analysis of cellular fatty acids (Pierson et al., 1992; Blanc et al., 1994; Stoakes et al., 1994; Thurm and Gericke, 1994; Lin et al., 1995). Advances in molecular biology over the past 10 years have opened new areas for microbial identification and characterization (Erlich et al., 1991; Mullis and Faloona, 1987; Persing, 1991; Saiki et al., 1988). Molecular biology techniques (for characterization of specific genes or gene segments) are now common in the clinical laboratories.

Brucella spp. are facultative intracellular bacteria that cause zoonotic disease of brucellosis worldwide to humans and animals (that is, cattle, goats, and pigs) leading to economic losses for the livestock industry. Detection of *Brucella* spp. takes 48 to 72 h (Kumar et al., 1997; Barrouin-Melo et al., 2007) that does not meet the rapid detection requirement of food industries. Due to the urgent need of fast, specific, sensitive and inexpensive

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method for the diagnosis of *Brucella* spp., Chen et al. (2013) developed LAMP method for its detection. According to WHO report, tuberculosis (TB) is second leading cause of death among infectious diseases worldwide after the human immunodeficiency virus (HIV) (WHO, "Global Tuberculosis Report," 2012). *Mycobacterium tuberculosis* is a slow-growing bacterium that needs 1 to 2 months for growing in a culture. Therefore, to control TB, a rapid and timely diagnosis of tuberculosis is essential to combat this disease. Kaewphinit et al. (2013) developed LAMP method for detection of *M. tuberculosis* bacteria from clinical sputum samples. Due to their rapidity and high sensitivity, such advanced molecular methods improve clinician's ability to interpret test results which further enable them to better customize their patient care. There are many articles covering importance of LAMP method as an effective diagnostic tool for infectious diseases (Notomi et al., 2000; Mori and Notomi, 2009; Fakruddin 2011; Saharan et al., 2014). This review is planned to study about the details of pathogenic bacteria detected by LAMP method.

WHY THERE IS NEED OF LAMP

Earlier, DNA hybridization studies were used to demonstrate relatedness among different bacteria. These technologies, however, like the determinations of phenotypic variables, are limited by microbial recovery and growth but then after few years, diagnostics using DNA-based tools, such as polymerase chain reaction (PCR), are increasingly popular due to their specificity and speed, as compared to culture-based methods (Louie et al., 2000). The amplified products, known as amplicons, may be characterized by various methods, including nucleic acid probe hybridization, analysis of fragments after restriction endonuclease digestion, or direct sequence analysis (Persing, 1991; Wagar, 2006). Further variations of PCR method like RT- PCR, ligase chain reaction (LCR), nested PCR, and multiplex PCR, etc have simplified and accelerated the process of nucleic acid amplification and easy detection of microbes (Wagar, 2006) but these all have drawbacks of less sensitivity, insufficient specificity, low amplification efficiency, not available for all species, high cost, use of special equipments etc. that is thermo cycler, complicated result detection methods, etc. So, there is a need of another powerful technique which can overcome all these drawbacks and this all became possible with LAMP.

Although the inception of loop-mediated isothermal amplification (LAMP) refers back to 1998, the popularity of LAMP starts only after 2003 following emergence of West Nile and SARS viruses. This technique was first described and initially evaluated for detection of hepatitis B virus DNA by Notomi et al. (2000). First of all, LAMP has been applied to many kinds of pathogens causing food-borne diseases (Lukinmaa et al., 2004). LAMP kits for detecting *Salmonella*, *Legionella*, *Listeria*, verotoxin-producing *Escherichia coli*, and *Campylobacter* have been commercialized. LAMP is a powerful and novel nucleic acid amplification method based on the principle of strand displacement activity that amplifies a few copies of target DNA with high specificity, efficiency, and rapidity under isothermal conditions, using a set of four specially designed primers and a DNA polymerase. The cycling reactions can result in the accumulation of 10^9 to 10^{10} fold copies of target in less than an hour (Notomi et al, 2000; Parida et al., 2008; Tomita et al., 2008). A large amount of product is formed, due to the strand displacement activity of *Bst* polymerase enzyme and because of this property; identification of a positive reaction does not require any special processing or electrophoresis (Mori et al., 2001). LAMP is isothermal which eradicates the need for expensive thermo cyclers used in conventional PCR; it may be a particularly useful method for infectious disease diagnosis in low and middle income countries (Macarthur, 2009).

LAMP METHODOLOGIES

Collection of bacterial strain

In LAMP method, infected blood samples from patients, infected food samples (fruit juices, various types of drinks, etc), sputum sample (in case of TB patients), urine and field samples (that is, collected directly from site of infection or from medical centers) can be used directly for detection of the pathogen.

Genomic DNA extraction from bacterial culture

There are a number of methods available that can be used to extract template for the LAMP process. These methods vary depending on the source material and whether RNA or DNA is required for the procedure. Commercial column based kits are most frequently used and have been used successfully for extraction from microbial cell cultures (En et al., 2008; Kubota et al., 2008; Tomlinson et al., 2007), animal tissue culture and from plant host species (Fukuta et al., 2003; Varga and James, 2006). However, a crude CTAB method has also been used to successfully extract the citrus greening organism from *Citrus* species (Okuda et al., 2005). Similar crude heat lysis methods have been used for many bacterial species (Savan et al., 2004; Song et al., 2005).

Design of primers for the LAMP method

DNA sequence is retrieved from NCBI (http://www.ncbi:nlm.nhi.gov/) and specific LAMP DNA oligonucleotide primers are designed from DNA sequence

using free online software that is, Primer-Explorer IV software program (http://venus.netlaboratory.com/partner/LAMP/pevl.html). The following four types of primers based on 6 distinct regions of the target gene; that is F3c, F2c and F1c regions at the 3' side and B1, B2 and B3 regions at the 5' side are to be designed:

i) F3 Primer: Forward Outer Primer consists of the F3 region that is complementary to the F3c region.
ii) B3 Primer: Backward Outer Primer consists of the B3 region that is complementary to the B3c region.
iii) FIP: Forward Inner Primer (FIP) consists of the F2 region (at the 3' end) that is complementary to the F2c region, and the same sequence as the F1c region at the 5' end.
iv) BIP: Backward Inner Primer (BIP) consists of the B2 region (at the 3' end) that is complementary to the B2c region, and the same sequence as the B1c region at the 5' end.

LAMP reaction

The LAMP reaction is carried out in a 25 µL reaction mixture containing 0.8 µM each of forward inner primer and backward inner primer, 0.2 µM each of F3 and B3, 400 µM each of deoxynucleoside triphosphate (dNTP), 1 M betaine, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 4 mM MgSO4, 0.1% Triton X-100, 8 U Bst DNA polymerase large fragment. 2 µl target DNA was added and mixture was incubated at 65°C for 1 h using a conventional heating block and then heated to 80° C for 10 min to terminate the reaction.

Mechanism of LAMP

The mechanism of the LAMP amplification reaction includes three steps: Production of starting material, cycling amplification, and recycling (Notomi et al., 2000; Tomita et al., 2008). Two inner and two outer primers are required for LAMP. In the initial steps of the LAMP reaction, all four primers are employed, but in the later cycling steps, only the inner primers are used for strand displacement DNA synthesis. The outer primers are referred to as F3 and B3, while the inner primers are forward inner primer (FIB) and backward inner primer (BIP). Both FIP and BIP contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA, one for priming in the first stage and the other for self-priming in later stages (Notomi et al., 2000). The size and sequence of the primers was chosen so that their melting temperature (Tm) is between 60 and 65°C, the optimal temperature for *Bst* polymerase. The final product in LAMP is a mixture of stem loop DNA with various stem length and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand (Notomi et al., 2000; Tomita et al., 2008).

Detection of amplified products

A number of methods are available that can be used for detection of products in LAMP method. Amplified products can be directly observed by the gel electrophoresis, naked eye or using a UV trans-illuminator, intercalating dyes like SYBR Green I stain, pitco green (Dukes et al., 2006; Iwamoto et al., 2003; Tomlinson and Boonham, 2008), by-products from the reaction chemistry (Goto et al., 2009) or by addition of hydroxyl-napthol blue, a chelating agent that changes colour due to the change in the concentration of Mg^{2+} ions (Goto et al., 2009).

Detection of amplicons or LAMP products

Naked eye: LAMP products can be directly observed by the naked eye in the reaction tube by adding 2.0 µl of 10 fold diluted SYBR Green I stain.

UV transilluminator: Under UV illumination, the gel shows a ladder-like structure.

Gel Electrophoresis: The result of LAMP reactions may be detected using gel electrophoresis.

Intercalating Dyes: The high specificity product produced during the LAMP process offers the use of intercalating dyes for amplification product detection. Intercalating dyes include SYBR green and Picogreen. Both dyes can be detected visually or by measurement in a real-time PCR machine or equivalent flourometer (Dukes et al., 2006; Iwamoto et al., 2003; Tomlinson and Boonham, 2008).

Chemical reactions: Two other alternatives that is, Magnesium pyrophosphate, which increases the turbidity of the reaction by precipitation, allowing the detection visually or more commonly, by spectrophotometer (Mori et al., 2011) and another is hydroxyl-napthol blue, a chelating agent that changes colour due to the change in the concentration of Mg^{2+} ions (Goto et al., 2009).

ADVANTAGES OF LAMP

A variety of pathogenic bacterial strains like *E. faecalis*, *M. ulcerans, M. tuberculosis, M. Pneumonia, S. typhi, B. anthracis* etc. were successfully identified by LAMP method developed by various researchers shown in Table 1.

Simplicity and cost-effectiveness

1) Isothermal - no need for thermal cycler,

Table 1. List of bacteria detected by LAMP assay till date.

Author's name	Organism name	Detection limit
Xu et al., 2014	*E. faecalis*	3.2 CFU/250 ml
Su et al., 2014	*S.aureus*	10 CFU/reaction
Kaewphinit et al., 2013	*M. tuberculosis*	5 pg
Lim et al., 2013	*S. aureus*	2.5 ng/µl
Wang et al., 2012 (a)	*S. agalactiae*	$2.8x10^3$ CFU/ml
Wang et al., 2012 (b)	*E. coli* various serogroups	10^3-10^4 CFU/ g
De Souza et al., 2012	*M. ulcerans*	48pg/µl
Tang et al., 2012	*S. enteric*	6.0CFU/test
Nagarajappa et al., 2012	Enterotoxigenic *Staphylococci*	100CFU/test
Yang et al., 2012	*Borrelia burgdorferi*	0.02-0.2pg
Sun et al., 2011	*V. parahaemolytics*	$2.4x10^2$ CFU/ml (pure), $8.9x10^2$CFU/ml (infected food sample).
Han et al., 2011	*V. vulnificus*	$2.5x10^3$ CFU/g
Kubota et al., 2011	*Ralstonia solanacearum*	10^4-10^6 CFU/ml
Kohan et al., 2011	*M. tuberculosis*	5 fg/reaction
Suwanampai et al., 2011	*S. aureus*	10^4 CFU/ml
Tang et al., 2011	*Listeria monocytogenes*	2.0 CFU/reaction
Lin et al., 2011	*Chlamydia psittaci* abortus strain	25 copies
Pan et al., 2011	*Brucella species*	10 pg (pure), 1.3x103 CFU/ml (contaminated milk).
Yang et al., 2011	*S. aureus*	1.25 CFU/reaction tube (pure), 10.3CFU/reaction tube (contaminated).
Ward et al., 2010	*Xylella fastidiosa*	200-25 0copies/reaction
Xu et al., 2010	*V. cholera*	25 CFU (pure), 32CFU (infected sample)
Techathuvanan et al., 2010	*S. typhimurium*	10^2-10^6 CFU/25g
Zhao et al., 2010	*S.* species	100 CFU/reaction
Fukasawa et al., 2010	*M. tuberculosis*	5,000 bacilli/ml sputum
Lu et al., 2010	*Legionella pneumophila*	576 fg (pure), 8CFU/ml (infected water sample).
Iseki et al., 2010	*Plasmodium knowlesi*	10^2-10^8 copies/µl
Nakao et al., 2010	*Ehrlichia ruminantium*	10 copies
Rigano et al., 2010	*Xanthomonas axonopodis* pv.*citri*	10 fg (pure), 18CFU (infected).
Kawai et al., 2009	*Chlamydophila pneumonia*	100%
Gahlawat et al., 2009	*Renibacterium salmoninarium*	10^{-8}
Yamazaki et al., 2010	*Vibrio paraharmolyticus* tdh and trh genes	0.8 CFU (tdh), 21.3CFU (trh-1), 5.0 CFU(trh-2).
Li et al., 2009	*Pseudomonas syringae pv. phaseolica*	$6.9x10^3$ CFU/ml
Hill et al., 2008	*Escherichia coli*	10 copies/reaction
Salah et al., 2008	*Renibacterium salmoninarum*	1 pg
Yamazaki et al., 2008 (a)	*Campylobacter jejuni*	5.6 CFU/g
Yamazaki et al., 2008 (b)	*V. cholerae*	$7.8x10^2$ CFU/g
Pandey et al., 2008	*M. tuberculosis*	100%
Misawa et al., 2007	Methicillin-resistant *S. aureus*	92.3%
Hara-kudo et al., 2007	*E. coli*	0.7 CFU/test
Qiao et al., 2007	*B. anthracis*	10 spores/tube (pure), 100spores/2mg powder (infected)
Boehme et al., 2007	Pulmonary tuberculosis	97.7%
Kato et al., 2007	*E. faecalis*	10 µg/tube
Aoi et al., 2006	Ammonia-oxidizing bacteri a	10^2 copies
El-Matbouli et al., 2006	*Thelohania contejeani*	10^{-5}
Kamachi et al., 2006	*Bordetella pertussis*	10 fg/DNA tube
Yeh et al., 2006	*Flavobacterium columnare*	30 pg/reaction tube
Mukai et al., 2006	*M. species*	500 copies

Table 1. Contd.

Ohtsuka et al., 2005	*S.enteric*	92.3%
Kato et al., 2005	*Clostridium difficile*	50 ng-0.5pg
Savan et al., 2005	Fish and shellfish pathogens	20 CFU
Hara-Kudo et al., 2005	*Salmonella*	2.2 CFU/test
Saito et al., 2005	*Mycoplasma pneumonia*	$2x10^2$ copies
Yeh et al., 2005	*Edwardsiella ictaluri*	20 CFU/ml
El-Matbouli et al., 2005	*Tetracapsuloides bryosalmonae*	100 folds more sensitive
Yoshida et al., 2005	*Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola*	1mg/tube(*P.gingivalis*),100fg/tube(*T.forsythia*),1mg/tube (*T. denticola*).
Seki et al., 2005	*S.. pneumonia*	10 copies
Maeda et al., 2005	*Porphyromonas gingivalis*	10^2-10^6 cells
Song et al., 2005	*Shigella* and enteroinvasive *Escherichia coli*	8 CFU/reaction
Horisaka et al., 2004	*Yersinia pseudotuberculosis*	10 CFU
Savan et al., 2004	Edwardsiellosis	$3.8X10^2$ CFU
Enosawa et al., 2003	*M. avium* subsp. para-tuberculosis	0.5-5 pg/tube
Iwamoto et al., 2003	*M. tuberculosis* complex	5-50 copies

g, gram; mg, milligram; ml, milliliter; pg, picogram; ng, nanogram; µl, microliter; µg, microgram; fg, femtogram; CFU, colony forming unit.

2) All required reagents are relatively cheap,
3) No need for excessive post-reaction handling steps.

Specificity

The use of six primers in LAMP provides a greater specificity than PCR. LAMP is less susceptible to interference (Notomi et al., 2000). LAMP is more specific than other techniques as many researchers have achieved even 100% specificity (Misawa et al., 2007; Tao et al., 2011; Wang et al., 2012; Yamazaki et al., 2008; Wang et al., 2010; Zhao et al., 2010) 97.3% specificity (Yeh et al., 2006), 95.9% (Pandey et al., 2008) and 94.2% (kohan et al., 2011) specificity.

Sensitivity

Many researchers have reported of achieving LAMP sensitivity as low as 6 copies/reaction for pure template. There is a general consensus that LAMP is 10 times more sensitive than standard PCR (En et al., 2008; Fukuta et al., 2003; Okuda et al., 2005; Savan et al., 2004; Dukes et al., 2006; Tomlinson and Boonham, 2008).

Rapidity

As the PCR and other methods proved to be time consuming, LAMP method is very fast and rapid. It can detect the infected bacteria; that is, generate results in an average of half an hour.

Direct use of sample from site of infection

In PCR and other molecular techniques for detection of pathogens, nucleic acid needs to be isolated but due to LAMP, it became possible to use directly the infected blood sample, food sample, sputum, urine samples directly from the site of infection. When compared to PCR, LAMP proves better than PCR in many ways as shown in Table 3. From all these, we can conclude that LAMP is a fast, rapid, economic, versatile and very valuable method and have emerged as a new era in the field of technology.

Lamp detection kits

Till now, a large number of bacterial pathogens have been detected by LAMP and still the research is going on but, after the detection of bacteria, some researchers have developed ready-made kits (Table 2) for more rapid and easier detection to be used at commercial level. These kits have all the reagents (thermopol buffer, betaine, dNTP's, primers, Bst polymerase enzyme, $MgSO_4$ in appropriate concentration) in it except, the nucleic acid sample which has to be added at the time of need. These ready-made kits have been commercialized by Eiken chemical company for detection of *M. tuberculosis* and Campylobacter spp. etc. (Eiken Chemical Co., Ltd. (Head office in Taito-ku, Tokyo).

SUMMARY AND FUTURE ASPECTS

No need for denaturing step in using the LAMP method. The whole amplification reaction takes place continuously

Table 2. LAMP based commercially available bacterial pathogen detection kits are listed below.

Year	Organism name	Name of kit
Mitarai et al., 2011	*M. tuberculosis*	Evaluation of a simple loop-mediated isothermal amplification test kit for the diagnosis of tuberculosis.
Eiken Chemical Co., Ltd., 2011	*M. tuberculosis*	Release of the “Loopamp® Tuberculosis Complex Detection Reagent Kit”, a pharmaceutical for *in vitro* diagnosis, as well as the “Loopamp® PURE DNA Extraction Kit”, “Loopamp® LF-160 Homeothermal Equipment with Fluorometer” and “PureLAMPTM Heater”
Eiken Chemical Co., Ltd., 2008	*M. tuberculosis*	“Loopamp TB detection Kit”
Eiken Chemical Co., Ltd., 2008	*C., Giardia*	Loopamp Cryptosporidium Detection Kit" and "Loopamp Giardia Detection Kit".
Eiken Chemical Co., Ltd., 2006	*Campylobacter*	"Loopamp *Campylobacter* detection Kit".
Eiken Chemical Co., Ltd., 2005	*E.coli O157*	Loopamp O157 detection Kit".
Eiken Chemical Co., Ltd., 2005	*L. monocytogenes*	Loopamp *L. monocytogenes* detection Kit".
Eiken Chemical Co., Ltd., 2004	*Legionella*	Loopamp *Legionella* screening Kit E" for environmental detection.
Eiken Chemical Co., Ltd., 2003	*Salmonella,* verotoxins	Novel Loopamp *Salmonella* screening kit, Loopamp verotoxin-producing *Escherichia coli* screening kit, and Loopamp Verotoxin Typing Kit.

Table 3. Comparison of PCR and LAMP.

Difference	PCR	LAMP
Definition	PCR is a rapid and simple technique of producing relatively large numbers of copies of DNA molecules from minute quantities of source DNA material	Loop-mediated isothermal amplification (LAMP) that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions
Denaturation step	Denaturation step is compulsory: Denature double stranded into a single stranded form	No need for a step to denature double stranded into a single stranded form
Specificity	Two primers are to amplify template DNA.	Four specially designed primers that recognize a total of six distinct sequences on the target DNA
Sensitivity	The sensitivity and specificity are not 100%	The sensitivity and specificity are 100%
Time requirement	PCR take more time than LAMP	LAMP take less time than PCR
Cost	Costly method in comparison to LAMP (5–7 $US per sample)	Cheapest method in comparison to PCR (about 70 cents US per sample)

under isothermal conditions. The amplification efficiency is extremely high. By designing 4 primers to recognize 6 distinct regions, the LAMP method is able to specifically amplify the target gene. The total cost can be reduced, as LAMP does not require special reagents or sophisticated equipments. The amplified products have a structure consisting of alternately inverted repeats of the target sequence on the same strand. Amplification can be done with RNA templates following the same procedure as with DNA templates, simply through the addition of reverse transcriptase.

LAMP method paves a new way to diagnose pathogenic microorganisms in clinical laboratories. It is compulsory to employ LAMP technique on large scale in resource-limited laboratories in developing countries, where many fatal tropical diseases are endemic. Also in near future, LAMP testing kits on readymade microchips are to be used by both developed and developing countries.

REFERENCES

Aoi Y, Hosogai M, Tsuneda S (2006). Real-time quantitative LAMP (loop-mediated isothermal amplification) of DNA as a simple method for monitoring ammonia-oxidizing bacteria. J. Biotechnol. 125(4):484-491.

Barrouin-Melo SM, Poester FP, Ribeiro MB, de Alcântara AC, Aguiar PH, Nascimento IL, Schaer RE, Nascimento RM, Freire SM (2007). Diagnosis of canine brucellosis by ELISA using an antigen obtained from wild *Brucella canis*. Res. Vet. Sci. 83:340-360.

Blanc DS, Lugeon C, Wenger A, Siegrist HH, Francioli P (1994). Quantitative antibiogram typing using inhibition zone diameters compared with ribotyping for epidemiological typing of methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. 32:2505-2509.

Boehme CC, Nabeta P, Henostroza G, Raqib R, Rahim Z, Gerhardt M (2007). Operational feasibility of using loop mediated isothermal amplification for diagnosis of pulmonary tuberculosis in microscopy center of developing countries. J. Clin. Microbiol. 45:1936-1940.

Chen S, Li X, Li J, Atwill ER (2013). Rapid detection of *Brucella* spp. using loop mediated isothermal amplification (LAMP). Methods. Mol. Biol. 1039:99-108.

De Souza DK, Quaye C, MosiIL, Addo P, Boakye DA (2012). A quick and cost effective method for the diagnosis of *Mycobacterium ulcerans* infection. BMC Infec. Dis. 12:8.

Dukes JP, King DP, Alexandersen S (2006). Novel reverse-transcription loop-mediated isothermal amplification for rapid detection of foot-and-mouth disease virus. Archives Virol. 151:1093-1106.

El-Matbouli M, Soliman H (2005). Rapid diagnosis of *Tetracapsuloides bryosalmonae,* the causative agent of proliferative kidney disease (PKD) in salmonid fish by a novel DNA amplification method, loop-mediated isothermal amplification (LAMP). Parasitol. Res. 96(5): 277-284.

El-Matbouli M, Soliman H (2006). Molecular diagnostic methods for detection of *Thelohania contejeani (Microsporidia)*, the causative agent of porcelain disease in crayfish. Dis. Aquat. Organ. 69(2-3):205-211.

En FX, Wei X, Jian L, Qin C (2008). Loop-mediated isothermal amplification establishment for detection of pseudorabies virus. J. Virol. Methods. 151:35-39.

Enosawa M, Kageyama S, Sawai K, Watanabe K, Notomi T, Onoe S, Mori Y, Yokomizo Y (2003). Use of loop-mediated isothermal amplification of the IS900 sequence for rapid detection of cultured *Mycobacterium avium* subsp. paratuberculosis. J. Clin. Microbiol. 41(9): 4359-4365.

Erlich HA, Gelfand D, Sninsky JJ (1991). Recent advances in the polymerase chain reaction. Science. 252:1643-1651.

Fakruddin M (2011). Loop mediated isothermal amplification-an alternative to Polymerase Chain Reaction (PCR). Bangladesh Res. Pub. J. 5:425-439.

Fukasawa T, Oda N, Wada Y, Tamaru A, Fukushima Y, Nakajima C, Suzuki Y (2010). A novel method for the purification of DNA by capturing nucleic acid and magnesium complexes on non-woven fabric filters under alkaline conditions for the gene diagnosis of tuberculosis by loop-mediated isothermal amplification (LAMP). Jpn. J. Infect. Dis. 63(4):246-250.

Fukuta S, Mizikami Y, Ishida A, Ueda J, Kanabe M, Ishimoto Y (2003).Detection of Japanese *yam mosaic virus* by RT-LAMP. Archives Virol. 148:1713-1720.

Gahlawat SK, Ellis AE, Collet B (2009).A sensitive loop-mediated isothermal amplification (LAMP) method for detection of *Renibacterium salmoninarum*, causative agent of bacterial kidney disease in salmonids. J. Fish Dis. 32(6):491-497.

Goto M, Honda E, Ogura A, Nomoto A, Hanaki K (2009). Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. Biotechniques. 46(3):167-172.

Han F, Wang F, Ge B (2011). Detecting potentially virulent *Vibrio vulnificus* strains in raw oysters by quantitative loop-mediated isothermal amplification. Appl. Environ. Microbiol. 77:2589-2595.

Hara-Kudo Y, Nemoto J, Ohtsuka K, Segawa Y, Takatori K, Kojima T, Ikedo M (2007). Sensitive and rapid detection of vero toxin-producing *Escherichia coli* using loop-mediated isothermal amplification. J. Medical Microbiol. 56:398–406.

Hara-Kudo Y, Yoshino M, Kojima T, Ikedo M (2005). Loop-mediated isothermal amplification for the rapid detection of *Salmonella*. FEMS Microbiol. Lett. 253(1): 155-161.

Hill J, Beriwal S, Chandra I, Paul VK, Kapil A, Singh T, Wadowsky RM, Singh V, Goyal A, Jahnukainen T, Johnson JR, Tarr PI, Vats A (2008). Loop-mediated isothermal amplification assay for rapid detection of common strains of *Escherichia coli.* J. Clin. Microbiol. 46(8):2800-2804.

Horisaka T, Fujita K, Iwata T, Nakadai A, Okatani AT, Horikita T, Taniguchi T, Honda E, Yokomizo Y, Hayashidani H (2004). Sensitive and specific detection of *Yersinia pseudotuberculosis* by loop-mediated isothermal amplification. J. Clin. Microbiol. 42(11):5349-5352.

Iseki H, Kawai S, Takahashi N, Hirai M, Tanabe K, Yokoyama N, Igarashi I (2010). Evaluation of loop-mediated isothermal amplification method as a tool for diagnosis of infection by the zoonotic simian malara parasite *Plasmodium knowlesi.* J. Clin. Microbiol. 48: 2509-2524.

Iwamoto T, Sonobe T, Hayashi K (2003). Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium*, and in sputum samples. J. Clin. Microbiol. 41:2616-2622.

Kaewphinit T, Arunrut N, Kiatpathomchai W, Santiwatanakul S, Jaratsing P, Chansiri K (2013). Detection of *Mycobacterium tuberculosis* by using loop-mediated isothermal amplification combined with a lateral flow dipstick in clinical samples.Hindawi Publishing Corporation BioMed Research International, Volume 2013, Article ID 926230, 6p.

Kamachi K, Toyoizumi AH, Toda K, Soeung SC, Sarath S, Nareth Y, Horiuchi Y, Kojima K, Akahashi M, Arakawa Y (2006). Development and evaluation of a loop-mediated isothermal amplification method for rapid diagnosis of *Bordetella pertussis* infection. J. Clin. Microbiol. 44(5):1899-1902.

Kato H, Yokoyama T, Kato H, ArakawaY (2005). Rapid and simple method for detecting the toxin B gene of *Clostridium difficile* in stool specimens by loop-mediated isothermal amplification. J. Clin. Microbiol. 43(12):6108-6112.

Kato H, Yoshida A, Ansai T, Watari H, Notomi T, Takehara T (2007). Loop-mediated isothermal amplification method for the rapid detection of *Enterococcus faecalis* in infected root canals. Oral Microbiol. Immunol. 22:131-135.

Kawai S, Hirai M, Haruki K, Tanabe K, Chigusa Y (2009). Cross-reactivity in rapid diagnostic tests between human malaria and zoonotic simian malaria parasite *Plasmodium knowlesi* infections. Parasitol. Int. 58:300-302.

Kohan L, Shahhosseiny MH, Razavi MR, Parivar K, Moslemi E, Werngren E (2011). Evaluation of loop mediated isothermal amplification for diagnosis of *Mycobacterium tuberculosis* complex in clinical samples. African J. Biotechnol. 10(26):5096-5101.

Kubota R, Schell MA, Peckham GD, Rue J, Alvarez AM, Allen C, Jenkins DM (2011). Bacterial and phytoplasma diseases in silico genomic subtraction guides development of highly accurate, DNA-based diagnostics for *Ralstonia solanacearum* race 3 biovar 2 and blood disease bacterium. J. General Pl. Pathol. 77(3):182-193.

Kubota R, Vine BG, Alvarez AM, Jenkins DM (2008). Detection of *Ralstonia solanacaerum* by loop-mediated isothermal amplification. Phytopathol. 98:1045-1051.

Kumar P, Singh DK, Barbuddhe SB (1997).Sero-prevalence of brucellosis among abattoir personnel of Delhi. J. Commun. Dis. 29:131-137.

Li X, Nie J, Ward L, Madani M, Hsiang T, Zhao Y, Boer SHD (2009). Comparative genomics-guided loop-mediated isothermal amplification for characterization of *Pseudomonas syringae* pv. Phaseolicola. J. Appl. Microbiol.107:717-726.

Lim KT, Teh CS, Thong KL (2013).Loop-mediated isothermal amplification assay for the rapid detection of *Staphylococcus aureus*. Biomed. Res. Int. Volume 2013 (2013), Article ID 895816, 5p. http://www.hindawi.com/journals/bmri/2013/895816/abs/

Lin D, Lehmann PF, Hamory BH, Padhye AA, Durry E, Pinner RW, Lasker BA (1995). Comparison of three typing methods for clinical and environmental isolates of *Aspergillus fumigatus.* J. Clin. Microbiol. 33:1596-1601.

Lin GZ, Zheng FY, Zhou JZ, Wang GH, Gong XW, Cao XA, Qiu QC (2011) Loop-mediated isothermal amplification assay targeting the omp25 gene for rapid detection of *Brucella spp.* Mol. Cell Probe. 25:126-129.

Louie M, Louie L, Simor AE (2000). The role of DNA amplification technology in the diagnosis of infectious diseases. CMAJ 163(3):301-9.

Lu QF, Zheng W, Luo P, Wu ZH, Li H, Shen JG (2010). Establishment of loop mediated isothermal amplification method for detection of *Legionella pneumophila*. J. Zhejiang Univ. (Medical Sciences). 39(3):305-310

Lukinmaa S, Nakari UM, Eklund M, Siitonen A (2004). Application of molecular genetic methods in diagnostics and epidemiology of food-borne bacterial pathogens. APMIS. 112:908-929.

Macarthur G (2009). Global health diagnostics: research, development and regulation. Academy of Medical Sciences Workshop Report.
Maeda H, Kokeguchi S, Fujimoto C, Tanimoto I, Yoshizumi W, Nishimura F, Takashiba S (2005). Detection of periodontal pathogen *Porphyromonas gingivalis* by loop-mediated isothermal amplification method. FEMS Immunol. Med. Microbiol. 43(2):233-239.
Misawa Y, Yoshida A, Saito R, Yoshida H, Okuzumi K, Ito N, Okada M, Moriya K, Koike K (2007). Application of loop-mediated isothermal amplification technique to rapid and direct detection of methicillin-resistant *Staphylococcus aureus (MRSA)* in blood cultures. J. Infect. Chemotherapy. 13(3):134-140.
Mitarai S, Okumura M, Toyota E, Yoshiyama T, Aono A, Sejimo A, Azuma Y, Sugahara K, Nagasawa T, Nagayama N, Yamane A, Yano R, Kokuto H, Morimoto K, Ueyama M, Kubota M, Yi R, Ogata H, Kudoh S, Mori T (2011). Evaluation of a simple loop-mediated isothermal amplification test kit for the diagnosis of tuberculosis. Int. J. Tuberc. Lung Dis. 15(9):1211-17.
Mori Y, Nagamine K, Tomita N, Notomi T (2001). Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. & Biophys. Res. Commun. 289:150-154.
Mori Y, Notomi T (2009). Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. J. Infect. Chemother. 15:62-69.
Mukai T, Miyamoto Y, Yamazaki T, Makino M (2006). Identification of *Mycobacterium* species by comparative analysis of the dnaA gene. FEMS Microbiol. Lett. 254(2): 232-9.
Mullis KB, Faloona FA (1987).Specific synthesis of DNA *in vitro* via a polymerase-catalyzed reaction. Methods Enzymol. 155: 335-350.
Nagarajappa S, Thakur MS, Manonmani HK (2012).Detection of enterotoxigenic *Staphylococci* by loop mediated isothermal amplification method. J. Food Safety. 32: 59–65.
Nakao R, Stromdahl EY, Magona JW, Faburay B, Namangala B, Malele I, Inoue N, Geysen D, Kajino KK, Jongejan F, Sugimoto C (2010). Development of loop-mediated isothermal amplification (LAMP) assays for rapid detection of *Ehrlichia ruminantium.* BMC Microbiol.10: 296.
Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000). Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28 (12): E63.
Ohtsuka K, Yanagawa K, Takatori K, Hara-Kudo Y (2005).Detection of *Salmonella enterica* in naturally contaminated liquid eggs by loop-mediated isothermal amplification, and characterization of *Salmonella* isolates. Appl. Environ. Microbiol. 71(11): 6730-6735.
Okuda M, Matsumoto M, Takana Y, Subandiah S, Iwanami T (2005).Characterisation of the tufB-secE-nusG-rplKAJL-rpoB gene cluster of the citrus greening organism and detection by loop-mediated isothermal amplification. Pl. Dis. 89: 705-711.
Pan W, Wang JY, Shen HY, Zhao MQ, Ju CM, Dong XY, Lin Yi L, Chen DJ (2011). Development and application of the novel visual loop-mediated isothermal amplification of Omp25 sequence for rapid detection of Brucella spp. J. Anim. Vet. Adv. 10(16): 2120-2126.
Pandey BD, Poudel A, Yoda T, Tamaru A, Oda N, Fukuswma Y, Lekhek B, chi T, Phetsuksiri B, Suzuki Y (2008). Development of an in-house loop-mediated isothermal amplification assay for detection of *Mycobacterium tuberculosis* and evolution in sputum samples of Nepalase patient. J. Med. Medical. 57: 439-443.
Parida M, Sannarangaiah S, Dash PK, Rao PV, Morita K (2008). Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. Reviews Medical Virol. 18: 407–421.
Persing DH (1991). Polymerase chain reaction: trenches to benches. J. Clin. Microbiol. 29: 1281-1285.
Pierson CL, Friedman BA (1992). Comparison of susceptibility to beta-lactam antimicrobial agents among bacteria isolated from intensive care units. Diag. Microbiol. Infect. Dis. 15: 19-30.
Qiao YM, Guo YC, Zhang XE, Zhou YF, Zhang ZP, Wei HP, Yang RF, Wang DB (2007).Loop-mediated isothermal amplification for rapid detection of *Bacillus anthracis* spores. Biotechnol. Lett. 29 (12): 1939-1946.
Rigano LA, Maran MR, Castagnar AP, Amarala AM, Vojnov AA (2010). Rapid and sensitive detection of citrus bacterial canker by loop-mediated isothermal amplification combined with simple visual evaluation method. BMC Microbiol.10: 176.
Saharan P, Khatri P, Dingolia S, Duhan JS, Gahlawat SK (2014). Rapid detection of viruses using loop-mediated isothermal amplification (LAMP): A Review. In R.K. Salar**,** S.K. Gahlawat, P. Siwach and J.S. Duhan (eds.). Biotechnology: Prospects and Applications Springer, New Delhi, Heidelberg, New York, Dordrecht, London, pp. 287-306. DOI 10.1007/978-81-322-1683-4
Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Sci. 239: 487-491.
Saito R, Misawa Y, Moriya K, Koike K, Ubukata K, Okamura N (2005). Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of *Mycoplasma pneumoniae*. J. Med. Microbiol. 54(11):1037-41.
Salah M, Soliman H, Matbouli ME (2008).Loop-mediated isothermal amplification for rapid detection of *Renibacterium salmoninarum* the causative agent of bacterial kidney disease. Dis. Aquat. Org. 81:143-151.
Savan R, Igarashi A, Matsuoka S, Sakai M (2004). Sensitive and rapid detection of edwardsiellosis in fish by a loop-mediated isothermal amplification method. Appl. Environ. Microbiol. 70(1):621-624.
Savan R, Kono T, Itami T, Sakai M (2005).Loop-mediated isothermal amplification, an emerging technology for detection of fish and shellfish pathogens. J. Fish Dis. 28(10):573-581.
Seki M, Yamashita Y, Torigoe H, Tsuda H, Sato S, Maeno M (2005). Loop-mediated isothermal amplification method targeting the lyt A gene for detection of *Streptococcus pneumoniae.* J. Clin. Microbiol. 43(4): 1581-1586.
Song T, Toma C, Nakasone N, Iwanaga M (2005). Sensitive and rapid detection of *Shigella* and enteroinvasive *Escherichia coli* by a loop mediated isothermal amplification method. FEMS Microbiol. Lett. 243:259-263.
Stoakes L, John MA, Lannigan R, Schieven BC, Ramos M, Harley D, Hussain Z (1994).Gas–liquid chromatography of cellular fatty acids for identification of *staphylococci.* J. Clin. Microbiol. 32:1908-1910.
Su J, Liu X, Cui H, Li Y, Chen D, Li Y, Yu G (2014).Rapid and simple detection of methicillin-resistance *staphylococcus aureus* by *orfX* loop-mediated isothermal amplification assay. BMC Biotechnol. 14:8.
Sun X, Xu Q, Pan Y, Lan W, Vivian CH (2011). A loop-mediated isothermal amplification method for rapid detection of *Vibrio parahaemolyticus* in seafood. Annals Microbiol. 62(1):263-271.
Suwanampai T, Pattaragulvanit K, Pattanamahakul P, Sutheinkul O, Okada K, Honda T, Thaniyavarn J (2011). Evaluation of loop mediated isothermal amplification method for detecting enterotoxin, a gene of *Staphylococcus aureus* in pork. Southeast Asian J. Trop. Med. Public Health: 42(6):1489-1497.
Tang MJ, Zhou S, Zhang XY, Pu JH, Ge QL, Tang XJ, Gao YS (2011). Rapid and sensitive detection of *Listeria monocytogenes* by loop-mediated isothermal amplification.Curr. Microbiol. 63(6):511-516.
Tang T, Cheng A, Wang M, Li X, He Q, Jia R, Zhu D,
Chen X (2012). Development and clinical verification of a loop-mediated isothermal amplification method for detection of *Salmonella* species in suspect infected ducks. Poult. Sci. 91(4):979-986.
Tao ZY, Zhou HY, Xia H, Xu S, Zhu HW (2011). Adaptation of a visualized loop-mediated isothermal amplification technique for field detection of *Plasmodium vivax* infection. Parasites Vect. 4(1):115.
Techathuvanan C, Draughon FA, D'Souza DH (2010). Loop-mediated isothermal amplification (LAMP) for the rapid and sensitive detection of *Salmonella typhimurium* from pork. J. Food Sci. 75(3):165-172.
Thurm V, Gericke B (1994). Identification of infant food as a vehicle in a nosocomial outbreak of *Citrobacter freundii*: epidemiological subtyping by allozyme, whole-cell protein, and antibiotic resistance. J. Appl. Bacteriol. 76: 553-558.
Tomita N, Mori Y, Kanda H, Notomi T (2008).Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat. Protocol. 3:877-882.
Tomlinson J, Barker I, Boonham N (2007). Faster, simpler, more-specific methods for improved molecular detection of *Phytophthora ramorum* in the field. Appl. Environ. Microbiol. 73:4040-4047.
Tomlinson J, Boonham N (2008). Potential of LAMP for detection of

plant pathogens. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources. 3:1-7.
Varga A, James D (2006).Use of reverse transcription loop-mediated isothermal amplification for the detection of Plum pox virus. J. Virol. Methods. 138:184-190.
Wagar EA (2006). Direct hybridization and amplification applications for the diagnosis of infectious diseases. J. Clin. Lab. Anal. 10:312-325.
Wang B, Cai SH, Lu YS, Zhang XL, Huang YC, Jian JC, Wu ZH (2012a). A loop-mediated isothermal amplification method targeting the fbsB gene for rapid detection of *Streptococcus agalactiae*, the causative agent of *Streptococcosis* in farmed fish. The Israeli J. Aquacult.-Bamidgeh. 64:7.
Wang F, Jiang L, Yang Q, Prinyawiwatkul W, Ge B (2012 b). Rapid and specific detection of *Escherichia coli* serogroups O26, O45, O103, O111, O121, O145, and O157 in ground beef, beef trim, and produce by loop-mediated isothermal amplification. Appl. Environ. Microbiol. 10:07975.
Wang L, Li Y, Chu J, Xu Z, Zhong Q (2010). Development and application of a simple loop-mediated isothermal amplification method on rapid detection of *Listeria monocytogenes* strains. Molecular Biol. Reports. 39(1):445-449.
Ward L, Harpe S, Clover G (2010). Development of a LAMP assay for *Xylella fastidiosa*. MAF Biosecurity New Zealand Technical Paper.
WHO, "Global Tuberculosis Report," 2012. http://www.who.int/tb/publications/global_report/gtbr12_main.pdf.
Xu X, Shuhong Z, Qingping W, Jumei Z, Fei L, Jianheng C (2014). Development and application of a loop-mediated isothermal amplification (LAMP) method for rapid and sensitive detection of *Enterococcus faecalis* in drinking water. J. Food Safety. 34: 103–110. http://loopamp.eiken.co.jp/e/lamp/
Xu Y, Li S, Li D, Zhang H, Jiang Y (2010). Rapid detection of *Vibrio cholerae* by loop mediated isothermal amplification (LAMP) method. Chinese J. Biotechnol. 26(3):398-403.
Yamazaki W, Kumeda Y, Misawa N, Nakaguchi Y, Nishibuchi M (2010). Development of a loop-mediated isothermal amplification assay for sensitive and rapid detection of the tdh and trh genes in *Vibrio parahaemolyticus* and related vibrio species. Appl. Environ. Microbiol. 76(3):820-888.
Yamazaki W, Seto K, Taguchi M, Ishibashi M, Inoue K (2008b). Sensitive and rapid detection of cholera toxin-producing *Vibrio cholerae* using a loop-mediated isothermal amplification. BMC Microbiol. 8:94.
Yamazaki W, Taguchi M, Ishibashi M, Kitazato M, Nukina M, Misawa N, Inoue K (2008a). Development and evaluation of a loop-mediated isothermal amplification assay for rapid and simple detection of *Campylobacter jejuni* and *Campylobacter coli.* J. Med. Microbiol. 57(4): 444-451.
Yang H, Ma X, Zhang X, Wang Y, Zhang W (2011). Development and evaluation of a loop-mediated isothermal amplification assay for the rapid detection of *Staphylococcus aureus* in food. Europ. Food Res. Technol. 232(5):769-776.
Yang J, Guan G, Niu Q, Liu Z, Li Y, Liu J, Ma M, Ren Q, Liu A, Luo J, Yin H (2012). Development and application of a loop-mediated lisothermal amplification assay for rapid detection of *Borrelia burgdorferi*. Transboundary & Emerging Dis. 10:1682-1865.
Yeh HY, Shoemaker CA, Klesius PH (2006).Sensitive and rapid detection of *Flavobacterium columnare* in channel catfish *Ictalurus punctatus* by a loop-mediated isothermal amplification method. J. Appl. Microbiol. 100(5):919-925.
Yoshida A, Nagashima S, Ansai T, Tachibana M, Kato H, Watari H, Notomi T, Takehara T (2005). Loop-mediated isothermal amplification method for rapid detection of the periodontopathic bacteria *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola*. J. Clin. Microbiol. 43(5):2418-24.
Zhao X, Wang L, Chu J, Li Y, Li Y, Xu Z, Li L, Shirtliff M E, He X, Liu Y (2010). Development and application of a rapid and simple loop-mediated isothermal amplification method for food-borne *Salmonella* detection. Food Sci. Biotechnol.19(6):1655-1659. http://loopamp.eiken.co.jp/e/lamp/ http://www.ncbi.nlm.nih.gov/

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Review

Advances in highly specific plant gene silencing by artificial miRNAs

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Endogenous microRNAs (miRNAs) are potent negative regulators of gene expression in plants and animals. Through artificially transformed miRNA (amiRNAs) to target one or several genes of interest is becoming a powerful tool for silencing genes. The characteristics and application prospect of artificial microRNA (amiRNA) technology were reviewed.

Key words: Gene-silence, RNA interference, MiRNA, Artificial-microRNA.

INTRODUCTION

Transgene-mediated gene silence through RNA interference (RNAi) offers a direct way of inactivating one or several specific genes (Small, 2007; Tang et al., 2007). RNAi transgenes are dominant and can be applied in many different genetic backgrounds for any known gene in the genome. The RNA interference effects were all acted through the small silencing RNAs (sRNAs) derived from the transcribed double-stranded RNA precursors (Watson et al., 2005; Sen and Blau, 2006). Some studies have systematically compared different silencing strategies and found that hairpin RNA interference (hpRNAi) produced more efficient silencing triggers than separately transcribed sense and antisense RNAs (Wesley et al., 2001; Chuang and Meyerowitz, 2000).

MicroRNAs (miRNA), which negatively regulate gene expression, are endogenous single-stranded small RNA molecules 21 to 23 nucleotides long. They were first discovered in the Victor Ambros Laboratory (Lee et al., 1993), but the term microRNA was first introduced in 2001 (Ruvkun, 2001). The miRNAs are processed by RNaseIII-like enzyme Dicer from short hairpin-loop structures known as miRNA precursors (premiRNA) that are derived from longer primary miRNA transcripts (Brodersen and Voinnet, 2006). In plants, miRNAs trigger target mRNA cleavage and destruction through perfect or near perfect base pairing (Moxon et al., 2008). Many reports the numerous protein–protein and protein–RNA interactions can Influence the regulation of miRNA metabolism and function. The studies have shown that both siRNAs and microRNAs can move out of their domain of expression, which also means alteration of several nucleotides that does not affect miRNA

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Abbreviations: amiRNA, Artificial microRNA; **RNAi**, RNA interference; **sRNAs**, small silencing RNAs; **hpRNAi**, hairpin RNA interference; **premiRNA**, miRNA precursors; **RdRP**, RNA-dependent RNA polymerase.

biogenesis (Chuck and O'Connor, 2010; Krol et al., 2010). For the plant breeder, modifying the endogenous miRNA precursors can target genes of interest with the generate artificial miRNA (amiRNA). So, gene discovery will certainly be accelerate by gene silence (Niu et al., 2006; Warthmann et al., 2013; Schwab et al., 2010). Artificial microRNA (amiRNA) is becoming a powerful tool for silencing genes in plants, and several amiRNA vectors have recently been developed based on the natural precursor structures. The amiRNA sequence does not have to be perfectly complementary to the target sites; it can be optimized to target only one or, alternatively, several sequence-related genes. In plants nucleus and cytoplasm some RNA cleavage can be guided by the small RNA (sRNA). If there are not much non-autonomous effects, a few of the stronger promoters may cause higher degrees of gene silence (Schwab et al., 2006; Eamens et al., 2011).

miRNA BIOSYNTHESIS

Most miRNA genes are far from protein genes in genetic distance and may have their own promoter and can be transcribed independently. Research found that when the host genes are expressing, large number of intronic miRNAs may not express. Whether it is expressed or not is dependent on the host gene promoter. The host genes contain significant fraction of miRNA genes in their introns with the same orientation and are thought to be co-processed from the host gene (Isik et al., 2010). miRNA first digest in nuclear within RNaseIII-Drosha, releasing a 60 to 80 nt intermediates with hairpin structure named miRNA precursor (Lee et al., 2002). miRNA precursor need to be transferred to the nucleus, then through the cytoplasm Dicer enzyme further processing, can become mature molecules. This process depends on the transfer mechanism---RanGTP/exportin-5 (Lund et al., 2004).

miRNA maturation process: First Dicer recognizes hairpin structural parts of miRNA precursor and digests two chains, then the rest of the precursor will be cut off, generating an incomplete pairs of small molecules of double-stranded RNA that has been phosphorylated at the 5'end and has a 2nt outstanding at the 3'end. Because of double-stranded RNA molecular thermodynamics instability, the chain miRNA * would degraded immediately. Almost all of the eukaryotic biological processes can be regulated by the miRNAs (microRNAs). The cell development and function sustaining can be dependent on the levels of miRNAs in their organism (Tran and Hutvagner, 2013).

ARTIFICIAL MICRORNA

AmiRNA technology refers to the use of miRNA expression characteristic, using endogenous miRNA precursor as expression framework, to produce small molecule RNA mediated gene silencing. The research showed that miRNAs biogenesis cannot be affected by the alteration of several nucleotides, which makes it possible to modify the endogenous miRNA precursors of target genes of interest by artificial miRNA (amiRNA). The amiRNAs have the high specificity to facilitate efficient gene silencing of the target gene(s) (Niu et al., 2006; Warthmann et al., 2013; Schwab et al., 2010). Recently, the natural precursor structures of ath-miR159a, ath-miR164b, ath-miR169d, ath-miR172a, ath-miR319a and osa-miR528 were frequently used. For example, Liu et al (2010) generated a simple amiRNA vector (pAmiR169d) based on the structure of Arabidopsis miR169d precursor (premiR169d), and Wang et al. (2010) established a highly efficient method for construction of rice artificial microRNA vectors based on the structure of precursor Osa-miR528.

ARTIFICIAL miRNA CONSTRUCTION METHOD

Warthmann et al (2008) established a miRNA design platform, WMD3 (Web MicroRNA Designer，http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) system, which can design artificial miRNA for more than 100 plants. By using "designer" of WMD3 tool, selecting the plant to interfere with the genome database and inputting target gene sequences as well as online submission, this system compares through the plant genome database (or EST databases) to prevent "miss", and according to the related amiRNA parameters (for example, Tm.) lists candidate amiRNA. WMD3 provides construction based on ath-miR319a, osa-miR528, pChlamiRNA2/3 as amiRNA template, PCR method to replace the carrier with miRNA clips (detailed method of constructs artificial miRNA can consult on the site).

Choosing appropriate candidate precursor

After a two-step selection process based on empirically established criteria for efficiency and specificity, suitable amiRNA candidates are improved as the knowledge on the biology of miRNAs grows (Schwab et al., 2006; Huntzinger and Izaurralde, 2011). Then, the microRNA sequences would add a 3' modification of nucleotides (de Alba et al., 2013). And the recent reports showed that the stability and efficiency of target repression of miRNA can be influenced by the 3' modifications (Wyman et al., 2011). Then, the design of amiRNA should meet the requirements that 5'end instability, the position 10 usually with an ''A'' base, and the considered amiRNA should have appropriate annealing temperature and free will.

Currently, 21mers from the reverse complement of the target transcripts are considered effective amiRNA candidates, if they have an ''A'' (sometimes also ''U'') at position 10 and display 5'end instability (higher AU con-

tent at the 5'end and higher GC content at the 3'end). The position 1 will be replaced by an ''U'' and all candidates then undergo a series of mutations at positions 13 to 15 and 17 to 21 followed by mappings against all currently known cDNA sequences or gene models for the particular species. Allowing two mismatch within two target genes at position 13 to 21 bases, Ossowski et al. (2008) think that in amiRNA 17 to 21 and target RNA existence 1 to 2 mismatch can prevent RdRP mediated sub-siRNA generated. When the target mRNA exist senior structure, amiRNA may be unable to achieve to this point. This can be prevented either by avoiding the use of mRNA advanced structural zone, or designing two artificial miRNAs to different target gene areas because of difficulty in predicting the senior mRNA structure in cells.

POLYCISTRONIC ARTIFICIAL MICRORNA

The polycistronic pri-miRNA can be generated by these clustered miRNAs which are found in close proximity to each other. A polycistronic pri-miRNA usually contains three miRNA, which can be processed by Drosha in the nucleus. Then, they would be transported to the cytoplasm where it is further processed into mature miRNA by Dicer (Farazi et al., 2013; Jain et al., 2012; Ouda and Fujita, 2013). Polycistronic amiRNA can target with many different genes, but whether the relative position of an amiRNA in the polycistronic pri-amiRNA transcript would affect its maturation and RNAi efficiency is the mainly concerned problem. The RNAi effects of these amiRNAs had been verified previously (Hu et al., 2009). Recently, Chen et al. (2010) inserted three amiRNA cassettes, which are against Fluc, EGFP, and lacZ reporter gene respectively, into the pDsRed vector in different orders. The results directly demonstrate that the maturation and function of an amiRNA is not apparently affected by its relative position in the multi-amiRNA expression vector. Chen et al. (2010) had also inserted a series of amiRNA cassettes into three major expression vectors in tandem, found that the number of concatenated amiRNA cassettes has an impact on the RNAi efficiency of single-copy amiRNA. At the same time, the researchers also found that, the three major expression vectors showed an apparently decreased inhibitory effect with an increasing number of more than four amiRNA cassettes.

PROSPECTS

AmiRNA with high efficiency and precision in gene function research area is an effective tool to replace hpRNAi. Almost all situations that use hpRNAi can use amiRNA alternatively. The amiRNA can also be used to study gene functions of multi-copy genes with existing complementary effect. AmiRNA can also foster antiviral plants, because the amiRNA mediated digestion of target genes allows partial bases mismatch and this can avoid gene silencing interference failure caused by virus gene variants. Much microrna can interact with some microbe in plants and multiple artificial microRNAs conferred robust resistance to the great mass of plant virus (Lafforgue et al., 2013; Kung et al., 2012; Balmer and Mauch-Mani, 2013). The mutant library is an important tool in the genome research. Arabidopsis and rice plants has been built by model T-DNA insertion mutant library, because of the T-DNA insertion preference and some genes are not inserted, many gene inserted mutants could never get. Development of amiRNA mutant library by the gene silencing can compensate for this shortcoming.

Conflict of Interests

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

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REFERENCES

Balmer D, Mauch-Mani B (2013). Small Yet Mighty – MicroRNAs in Plant-Microbe Interactions. MicroRNA 2(1):73-80.

Brodersen P, Voinnet O (2006). The diversity of RNA silencing pathways in plants. Trends Genet. 22:268–280.

Chen P, Hu T, Fu Q, Liu Y, Ishaq M, Li J (2010).Comparative studies of various artificial MicroRNA expression vectors for RNAi in mammalian Cells. Mol. Biotechnol. 46:34–40.

Chuang CF, Meyerowitz EM (2000). Specific and heritable genetic interference by double-stranded RNA in Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA. 97:4985–4990.

Chuck G, O'Connor D (2010). Small RNAs going the distance during plant development. Curr. Opin. Plant Biol. 13:40–45.

de Alba AEM, Parent JS, Vaucheret H (2013). Small RNA-Mediated Control of Development in Plants. Epigenetic Memory and Control in Plants 18:177-199

Eamens AL, Agius C, Smith NA, Waterhouse PM, Wang MB (2011). Efficient Silencing of Endogenous MicroRNAs Using Artificial MicroRNAs in Arabidopsis thaliana. Mol. Plant 4:157–170.

Farazi TA, Hoell JI, Morozov P, Tuschl T (2013). MicroRNAs in Human Cancer. MicroRNA Cancer Regulation 774:1-20.

Huntzinger E, Izaurralde E (2011). Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat. Rev. Genet. 12:99-110

Hu T, Fu Q, Chen P, Ma L, Sin O (2009). Construction of an artificial MicroRNA expression vector for simultaneous inhibition of multiple genes in mammalian cells. Int. J. Mol. Sci. 10:2158–2168.

Jain AK, Allton K, Iacovino M, Mahen E, Milczarek RJ, Zwaka TP, Kyba M, Barton MC (2012). p53 Regulates Cell Cycle and MicroRNAs to Promote Differentiation of Human Embryonic Stem Cells. PLoS Biol. 10(2):e1001268.

Krol J, Loedige I, Filipowicz W (2010). The widespread regulation of microRNA biogenesis, function and decay. Nat. Rev. Genet. 11:597-610.

Kung YJ, Lin SS, Huang YL, Chen TC, Harish SS, Chua NH, Yeh SD

(2012). Multiple artificial microRNAs targeting conserved motifs of the replicase gene confer robust transgenic resistance to negative-sense single-stranded RNA plant virus. Mol. Plant Pathol. 13(3):303-317.
Lafforgue G, Martínez F, Niu QW, Chua NH, Daròs JA, Elena SF (2013). Improving the Effectiveness of Artificial MicroRNA (amiR)-Mediated Resistance against Turnip Mosaic Virus by Combining Two amiRs or by Targeting Highly Conserved Viral Genomic Regions. J. Virol. 87(14):8254-8256.
Lee RC, Feinbaum RL, Ambros V (1993). The C. elegans hetero chronic gene lin-4 encodes small RNAs with antisense comple-mentarity to lin-14. Cell 75:843–854.
Lee Y, Jeon K，Lee JT (2002). MicroRNA maturation: stepwise processing and sub-Cellular alization. EMBO J. 2l: 4663-4670.
Liu C, Zhang L, Sun J, Wang MB, Liu Y (2010). A simple artificial microRNA vector based on ath-miR169d precursor from Arabidopsis. Mol. Biol. Rep. 37:903–909.
Isik M, Korswagen HC, Berezikov E (2010). Expression patterns of intronic microRNAs in Caenorhabditis elegans. Science 1(1):1-9.
Lund E, Suttinger S, Calado A (2004). Nuclear export of microRNA precursors. Science 303:95-98.
Moxon S, Jing R, Szittya G, Schwach F, Moulton V (2008). Deep sequencing of tomato short RNAs identifies microRNAs targeting genes involved in fruit ripening. Genome Res. 18:1602–1609.
Niu QW, Lin SS, Reyes JL, Chen KC (2006). Expression of artificial microRNAs in transgenic Arabidopsis thaliana confers virus resistance. Nat. Biotechnol. 24:1420–1428.
Ossowski S，Schwab R，Weigel D (2008). Gene silencing in plants using artificial microRNAs and other small RNAs. Plant J. 53:674–690.
Ouda R, Fujita T (2013). Antiviral MicroRNA. Chembiomolecular Science 201-205.
Ruvkun G (2001). Glimpses of a tiny RNA world. Science 294:797–799.
Schwab R, Ossowski S, Warthmann N, Weigel D (2010). Directed Gene Silencing with Artificial MicroRNAs. Methods in Molecular Biology. 592:71-88
Schwab R, Ossowski S, Riester M, Warthmann N, Weigel D (2006). Highly specific gene silencing by artificial microRNAs in Arabidopsis. Plant Cell. 18:1121–1133.
Sen GL, Blau HM (2006). A brief history of RNAi: the silence of the genes. FASEB J. 20:1293–1299.
Small I (2007). RNAi for revealing and engineering plant gene functions. Curr. Opin. Biotechnol. 18:148–153.
Tang G, Galili G, Zhuang X (2007). RNAi and microRNA: breakthrough technologies for the improvement of plant nutritional value and metabolic engineering. Metabolomics 3:357–369.
Tran N, Hutvagner G (2013). Biogenesis and the regulation of the maturation of miRNAs. Essays Biochem. 54(1):17-28.
Wang XM, Yang Y, Yu CL Zhou J, Cheng Y (2010).A Highly Efficient Method for Construction of Rice Artificial MicroRNA Vectors. Mol. Biotechnol. 46:211-218.
Warthmann N, Ossowski S, Schwab R, Weigel D (2013). Artificial MicroRNAs for Specific Gene Silencing in Rice. Methods Mol. Biol. 956:131-149
Warthmann N, Chen H, Ossowski S, Weigel D, Herve P (2008). Highly specific gene silencing by artificial miRNAs in rice. PLoS One 3:e1829.
Watson JM, Fusaro AF, Wang M, Waterhouse PM (2005). RNA silencing platforms in plants. FEBS Lett. 579:5982–5987.
Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT (2001). Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J. 27:581–590.
Wyman SK, Knouf EC, Parkin RK, Fritz BR, Lin DW, Dennis LM, Krouse MA, Webster PJ, Tewari M (2011). Post-transcriptional generation of miRNA variants by multiple nucleotidyl transferases contributes to miRNA transcriptome complexity. Genome Res. 21(9):1450-1461.

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Review

Indigenous leafy vegetables (*imifino*, *morogo*, *muhuro*) in South Africa: A rich and unexplored source of nutrients and antioxidants

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South Africa is endowed with agro-biodiversity that consists of different types of indigenous leafy vegetables (ILVs) with health benefits and rich source of nutrients to cater for over three million people suffering from hunger and malnutrition in the country. Unfortunately, the use of these vegetables is declining at an alarming rate due to negligence and lack of appropriate cultivation practices to improve yield, quality and adaptability of valuable species. The nutritional value and antioxidant potential arising from their rich polyphenolic constituents are described in this review as useful inexpensive resources for reducing hidden hunger, prevention and control of cancer, hypertension, obesity, diabetes and heart disease. A total number of 22 plant species belonging to 12 genera and 10 families have been reviewed. *Amaranthus* species, *Cucurbita pepo*, *Bidens pilosa*, *Chenopodium album* and *Solanum nigrum* appear to be the most popular and most widely occurring leafy vegetables in the rural areas of South Africa. There is a need to create an atmosphere of awareness that would encourage consumption and industrial production of these vegetables in a bid to curb the high level of malnutrition and food insecurity in South Africa.

Key words: Indigenous leafy vegetables, antioxidants, nutritional value, food security, non communicable diseases, South Africa.

INTRODUCTION

Traditional African leafy vegetables are underutilized in South Africa (Voster and van Rensburg, 2005; Mnkeni et al., 2007). They are called *imifino* in isi Zulu or isi Xhosa, *morogo* in Sesotho or isi Pedi and *muhuro* in Tshivenda (van Rensburg et al., 2007; van der Walt et al., 2009). These groups of plants are neglected and despised despite their rich nutrient and mineral content including proteins, carbohydrates, vitamins and dietary fibre which are beneficial in the maintenance of good health and prevention of diseases (Nnamani et al., 2009). They are classified in the Eastern Cape Province and other parts of the country as 'poor peoples' food and knowledge associated with them is referred as 'backward knowledge' leading to an unwillingness of the youth to be associated with these crops (Voster and van Rensburg, 2005). Consequently, knowledge regarding their habitat and importance is hardly being transferred to the younger generation due to their changing social values and

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migration from the rural areas where these crops are consumed to the cities where they are ignored and neglected in favour of exotic western varieties (van Rensburg et al., 2004; van der Walt et al., 2009). There is therefore the need to collect, preserve and document this knowledge which can be useful for crop improvement and maintenance of local cultures and traditions. Chemical elimination of these vegetables, which are often considered as weeds, also makes their survival precarious, resulting in the loss of valuable species (Shackleton, 2003; Lewu and Mavengahama, 2010). In South Africa, just like in many other countries, most human plant food is based on a rather limited number of crops (Misra et al., 2008). This places the national/global food supply and economy at risk of collapse should there be a crisis with the few crops selected for human consumption (Mlakar et al., 2010). The neglect of indigenous leafy vegetables (ILVs) in South Africa is not proper given that the nutritional value of traditional leafy vegetables in most parts of the world has been reported to be higher than several known common vegetables (Sundriyal and Sundriyal, 2001; Ndlovu and Afolayan, 2008).

Indigenous leafy vegetables are usually not available on a commercial scale. However, they have the potential for income generation but fail to compete with exotic varieties due to lack of awareness. Most of the crops are not cultivated, but grow in the wild where they are well adapted to harsh environmental conditions (Lewu and Mavengahama, 2010; Matenge et al., 2012). In fact, some varieties such as *Amaranthus* and *Bidens pilosa* are known to be resistant to pest and diseases and therefore may constitute good sources of genes for genetic improvement of other crop varieties (Adebooye et al., 2004; Mnkeni et al., 2007).

Despite their rich nutrient content and adaptability to harsh climatic conditions, few scientific studies have been done to establish a seed and germplasm for African leafy vegetables and to determine their role and importance in the formulation of healthy diets in South Africa. This is surprising considering that almost 239 million people are suffering from hunger in sub-Saharan Africa, a figure that is likely to increase in the near future (Sasson, 2012). Twenty eight percent of children under the age of five years in Sub-Saharan Africa are moderately or severely underweight, an indicator for protein energy malnutrition (UNICEF, 2006). Close to 190 million young children and more than 15 million pregnant women in the developing world are vitamin A-deficient (WHO, 2009). Many authors have reported scarcity of vegetables in diet as a major cause of this deficiency, which may result in blindness in young children within the semi-arid and arid areas of Africa (Nojilana et el., 2007; Seidu et al., 2012; Nyuar et al., 2012).

In South Africa, most provinces are still challenged by high levels of poverty, especially among rural communities and in some areas the poverty level is as high as 78.2% (Lewu and Mavengahama, 2010). The rate of vitamin A deficiency is also high in the country (Labadarios, 2005). Sixty four percent of 1-9-year old children are vitamin A-deficient, 28% anaemic, 13% have poor iron levels and 45% had low zinc levels (Faber et al., 2011).

More than 40% of the adult population in South Africa is either overweight or obese with malnutrition being the predominant contributor as many people tend to consume processed foods that are high in saturated fats, sugar and salt (Puoane et al., 2002; Faber et al., 2011). On the other hand, consumption of vegetables is generally associated with reduced risk of cardiovascular diseases, cancer, stroke and reduced mortality (He et al., 2006). It is also worth mentioning that the deficiency of one micronutrient can exacerbate the deficiency of another, thus there is likely to be concomitant deficiencies of more than one micronutrient in many of South Africa's undernourished children (Black, 2003; Uusiku et al., 2010). These problems can be prevented by the inclusion of indigenous leafy vegetables like 'umifino umtyuthu', 'cetshana' or 'ityabontyi' (*Amaranthus cruentus*, *Cucurbita pepo*, *Citrillus lanatus*) in the diet as natural and inexpensive sources of vitamin A, iron and zinc. According to Uusiku et al. (2010) and based on the recommended nutrient intake (RNI), 300 g fresh ILVs would fulfil the dietary requirements of vitamin A for children. For adults, 300 g of fresh *Cucurbita pepo* would contribute 116% of female RNI and 97% of males RNI, whereas 300 g of fresh *Vigna unguiculata* would contribute 59% and 50% of female and male daily requirements, respectively.

A recent study on eight African leafy vegetables by van Jaarsveld et al. (2014) also reveals that pigweed and cowpea leaves are good sources of vitamin A, able to meet more than 75% of the recommended daily allowance (RDA) in children followed by spider flower, black night shade, tsamma melon, Jews mallow and pumpkin leaves (50% to 75% RDA). Studies carried out by Faber et al. (2007) also indicated that consumption of dark-green leafy vegetables contributed significantly to the dietary intake of calcium, iron, vitamin A and riboflavin in children in two rural villages of KwaZulu-Natal. There is therefore the dire need to encourage sufficient production, availability and consumption of indigenous leafy vegetables in a bid to curb the problem of malnutrition, obesity, food insecurity and poverty in the country. This review examines the nutritional and medicinal potentials of indigenous leafy vegetables in South Africa in an attempt to create awareness about their roles in the maintenance of good health and prevention of diseases.

COMMON TYPES OF ILVS

The Agricultural Research Council, South Africa has documented numerous types of leafy vegetables in the country including *Amaranthus*, *Brassica*, *Solanum*, *Chorchorus*, *Chenopodium* species and many others (Kleynhans et al., 2013). However, only the most common

varieties are described in this review.

Chenopodium album

Known as lamb's quarters, the leaves and young shoots of *C*. *album* are used as vegetables in some rural settings in the Eastern Cape Province and other parts of South Africa (Gqaza et al., 2013). *C*. *album* like with many other indigenous leafy vegetables in the country is usually not cultivated but harvested from the wild or roadside paths where it grows as a weed (Gqaza et al., 2013). It is known as *Imbikicane* in isi Xhosa and is usually prepared in combination with maize as porridge. It is an erect annual herb that may grow to a height of up to 1m (Singh et al., 2011). The stems are angular, ribbed with longitudinal dark green or red streaks. *C*. *album* belongs to the family Chenopodiaceae which consist of about 21 species, including *C*. *botrys*, *C*. *ambrosioides*, *C*. *murale*, *C*. *chilense* and *C*. *amaranticolor*, many of them with medicinal properties (Yadav et al., 2007). *C*. *quinoa* is also used as pseudo cereal in Bolivia and some South American countries (Alvarez-Jubete et al., 2010). They have an under-exploited potential to contribute to food security, nutrition, health and income generation in South Africa.

Portulaca oleracea

P. *oleracea* is commonly referred to as purslane in English, *igwanitsha* in isiXhosa and *amalenyane* in isiZulu (Dweck, 2001). It is a green vegetable with succulent stems and leaves with rich mucilaginous substance. It grows in soils with less water and nutrients, producing yellow flower buds. It grows to a height of 12 to 15 cm as a low-lying creeper, leaves and stems are tender to touch. It belongs to the family Portulacaceae which consist of about 150 species including *P*. *quadrifida*, *P*. *afra*, *P*. *pilosa*, *P*. *insularis* and *P*. *psammotropha* (Chung et al., 2008). It is eaten as a salad and vegetable all around the world and used medicinally for a variety of conditions that include headache, stomach ache, painful urination, enteritis, mastitis, lack of milk flow in nursing mothers and in postpartum bleeding (Dweck, 2001). In some rural settings in South Africa, the succulent weed is a favourite vegetable. Children eat the leaves raw (Dweck, 2001).

Amaranthus species

The genus *Amaranthus* is made of approximately 60 species most of which are cosmopolitan weeds (*Amaranthus retroflexus*, *Amaranthus hybridus*, *Amaranthus powellii* and *Amaranthus spinosus*.) and cultivated amaranth species (*Amaranthus blitum*, *Amaranthus lividus*, *Amaranthus viridis*, *Amaranthus gracilis*, *Amaranthus tricolor*, *Amaranthus gangeticus*, *Amaranthus hypochondriacus* and *Amaranthus thunbergii*) which can be used as food grain, leafy vegetables, forage and ornamentals (Mlakar et al., 2010). They appear as erect plant annuals or short-lived perennials and may grow to a height of 2 m. The mature vegetable amaranths produce tiny shiny seeds that are dark brown to black as opposed to cream-coloured seeds in the grain types (van Rensburg et al., 2007). They belong to the family Amaranthaceae, sub-family Amaranthoideae. The name 'amaranth' signifies 'immortal', 'everlasting' or 'non-wilting' in Greek (Mlakar et al., 2010), which is consistent with its ability to thrive in minimally nutritive soils and harsh environmental conditions. This group of plants has raised a lot of interest among researchers in many European countries because of their high nutrient quality, particularly associated with the grains. Their spinach-like flavour, high yields and ability to grow in hot weather have made them popular vegetable crops in most parts of Africa and Asia (van der Walt et al., 2009). Vegetable amaranths are the most popular and the most widely occurring leafy vegetables in many rural areas in South Africa where they appear as weeds, commonly referred to as pig weed in English and *unomdlomboyi* in isi Xhosa (Modi, 2007).

Bidens pilosa

Also known as black jack, *B*. *pilosa* is a cosmopolitan weed widely distributed in many parts of South Africa and other sub-tropical and tropical countries (Bartolome et al., 2013). It is an annual aggressive plant that may grow to a height of 1m. It flowers, producing white petals on small heads, barbed awns and fruits that easily catch on to animal fur and human clothing, a very effective means of seed dispersal. It belongs to the family Asteraceae which consist of about 240 species (Arthur et al., 2012). Just like most other weeds, *B*. *pilosa* is endowed with a remarkable ability to thrive in minimally nutritive soils. The young tender shoots are used as vegetable in many rural areas in Africa particularly in times of food scarcity. In some rural areas of South Africa, the bitter taste of this vegetable is a delicacy particularly among men who consume it in a mixture of other leafy vegetables (Voster and van Rensburg, 2005). Consumption of the leaves has been reported as a risk factor for oesophageal cancer in South Africa (Arthur et al., 2012). Traditional processing methods which may require boiling and squeezing to remove excess fluids may reduce to safety levels, some of the carcinogenic components. Pharmacological studies of this plant have revealed the presence of many bioactive compounds including terpenes, tannins, essential oils, amino acids and ascorbic acid (Silva et al., 2011). These findings are consistent with its folkloric uses in the treatment of gastrointestinal diseases by the Zulu tribe of South Africa (Voster and van Rensburg, 2005; Arthur et al., 2012).

Solanum nigrum

S. *nigrum* is called black nightshade in English and *Umsobo* in isi Xhosa (van Rensburg et al., 2007). It is an

erect annual or biannual herbaceous plant and may sometimes be perennial. It can reach up to 100 cm in height (Akubugwo et al., 2007a). The stem may be smooth or bear small hairs known as trichomes. These plants are widely distributed in South Africa and many other African countries where they grow as weeds in arable lands, gardens and soils rich in nitrogen. *S. nigrum* belongs to the family Solanaceae, a cosmopolitan family containing many essential vegetables and fruits such as tomatoes, aubergines, paprika, chillies, green and red peppers and cape goose berries, as well as ornamentals such as *Petunia, Schizanthus* and *Lycium* species (Edmonds and Cheweya, 1997). There are more than 1500 *Solanum* species, many of which are also economically important throughout their cosmopolitan distribution. The leaves are alternate and bright green in colour but purple pigmentation may be present (van Rensburg et al., 2007). The plant produces small flowers that are about 4 to 10 mm long with white petals and conspicuous yellow anthers that are arranged in a drooping umbel-like inflorescence. Leaves and tender shoots are widely used as vegetables throughout the world and have provided a food source since early times. In South Africa, *S. americanum*, *S. nigrum* and *S. retroflexum* are the most commonly used species (van Rensburg et al., 2007). Most of these vegetables are harvested from the wild and usually not domesticated. These plants are also believed to be medicinal especially against ulcers, toothache and swellings (Edmonds and Cheweya, 1997; Maanda and Bhat, 2010). The leaves in particular contain relatively high levels of oxalate and cyanide, but the processing and cooking done prior to consumption reduces the content of these bitter and potentially toxic compounds (Maina and Mwangi, 2008).

Cleome gynandra L.

C. gynandra is known as spider flower or cats whiskers in English and *amazonde* in isiZulu. It belongs to the family Capparaceae and grows as a weed in common barren land, road sides, open grass lands and crop fields in many parts of the world (van Rensburg et al., 2007; Mishra et al., 2011). It is widespread in Southern Africa extending to Limpopo, North West, Gauteng, Mpumalanga, KwaZulu-Natal, Free State, the Northern Cape and Namibia (Mishra et al., 2011). It is an erect annual herb, 250 to 600 mm tall; much branched and sometimes may become woody with age (Mishra et al., 2011). The leaves are palmately compound with three to five leaflets. The leaf stalk is 20 to 50 mm long with glandular hairs. When the plant flowers, it produces white petals, sometimes fading to rose pink, 20 to 20 × 3 to 5 mm, rounded at the apex and abruptly narrowed to a basal claw (Mishra et al., 2011). Other species which are occasionally used as vegetables include *Cleome hirta* and *Cleome monophylla* (van Rensburg et al., 2007).

Their leaves and the tips are harvested and used as a vegetable in the northern part of South Africa and generally preferred to vegetable amaranth (van Rensburg et al., 2007).

Corchorus species

The main species include *Corchorus olitorius*, *Corchorus tridens*, *Corchorus asplenifolius* and *Corchorus trilocularis* (van Rensburg et al., 2007). The English name is Jew's Mallow. These slimy vegetables are mostly consumed among rural communities in the northern parts of South Africa including Limpopo, Gauteng and Mpumalanga provinces (Ndlovu and Afolayan, 2008). They are known as *delele* in Tshivenda. *Corchorus* belongs to the family Tiliaceae and is an erect annual herb that varies from 20 cm to approximately 1.5 m in height (van Rensburg et al., 2007; Maanda and Bhat, 2010). The plants are usually harvested from the wild but have the potential to be developed into valuable crops. Very little is known about their role in the overall food acquisition system in different parts of South Africa especially in relation to their contribution to the intake of important micronutrients (Ndlovu and Afolayan, 2008).

Pumpkin and melon leaves

The leaves of "ordinary" pumpkin (*Cucurbita pepo*, *Cucurbita moschata* and *Cucurbita maxima*) and bitter melon (*Citrillus lanatus*) are widely consumed in many parts of Africa. In South Africa, *C. lanatus* is occasionally cultivated as a minor crop in maize fields but most other species are harvested from the wild (van Rensburg et al., 2007). They belong to the family Cucurbitaceae which consist mainly of melons, watermelons, various gourds and pumpkins (Maanda and Bhat, 2010). The seeds of *C. lanatus*, a creeping annual herb with hairy stems and leaves spiny to touch are also widely consumed in some West African countries (Ojieh et al., 2007).

Brassica rapa

This is the non-heading type of Chinese cabbage, an annual flowering vegetable with dark green leaves supported by light green to white petioles that form a rosette (van Averbeke et al., 2007). It is known as *Isiqwashumbe* in isiXhosa and *mutshaina* in Tshivenda. It is a common plant in the Vhembe district, north of the Limpopo province of South Africa (van Rensburg et al., 2007). It belongs to the family Brassicaceae or Cruciferae. It has a stout taproot and may grow to a height of 15 to 30 cm (van Averbeke et al., 2007).

NUTRITIONAL COMPOSITION OF ILVS

Indigenous leafy vegetables constitute an inexpensive source of macronutrients (fibre, starch, proteins and fats) and micronutrients (vitamins and minerals) (Odhav et al.,

2007; Makobo et al., 2010; Kwenin et al., 2011), with variations in quantities among families, genera and species (Table 1). The leaves of *Amaranthus* for example have been reported to contain 17.5 to 38.3% dry matter as protein of which 5% is lysine (Mnkeni et al., 2007). Both essential and non essential amino acids are represented in different species of *Amaranthus* in varying amounts. In a study on the nutritional composition of *Amaranthus hybridus*, Akubugwo et al. (2007b) reported 41.1% abundance for isoleucine, leucine, lysine, methionine, cysteine, phenylalanine, tyrosine, threonine, valine and 58.9% for the nonessential amino acids. Other authors have also made similar observations (Aremu et al., 2006; Hassan and Umar, 2006). Many studies carried out in South Africa have also documented the high nutrient content of local vegetables including *Corchorus olitorius*, *Cleome gynandra*, *Cleome monophylla* and *Solanum nigrum* (Mnkeni et al., 2007; Ndlovu and Afolayan, 2008; Akula and Odhav, 2008; van der Walt et al., 2009). In one such study, Ndlovu and Afolayan (2008) reported that the magnesium content of *C*. *olitorius*, a locally consumed vegetable was higher than cabbage (*Brassica oleraceae*) and spinach (*Spinacea oleracea*). This is an indication that its consumption might help meet the daily requirements of this mineral and many others especially in African rural settings where the consumption of micronutrient-deficient starchy staples is common place (Uusiku et al., 2010). However, anti-nutritional factors such as cyanogenic glycosides, oxalate, phytate, saponins and tannins have been reported in some African leafy vegetables (Kumari et al., 2004; Uusiku et al., 2010; Umar et al., 2011; Aregheore, 2012). Some of these compounds may affect the palatability of the species or pose a health hazard when consumed in large quantities. There is therefore the need for empirical studies that would shed more light on the safety parameters as well as the mutagenic potentials of African leafy vegetables. However, there is also the general believe that some of the anti-nutritive factors may contribute to the medicinal potentials of these vegetables and therefore are important as well. In addition, the very laborious and time-consuming traditional processing methods used in processing vegetables in most African settings may eliminate or reduce to safety levels many of the anti-nutritive factors (Aregheore, 2012).

VITAMIN-COMPOSITION OF ILVs

Vitamins A and C

Indigenous leafy vegetables are a rich source of vitamin A which occurs as provitamin A carotenoids such as lutein, α- γ- or β-carotene, violaxanthin and neoxanthin (Uusiku et al., 2010; van Jaarsveld et al., 2014)). However, the bioavailability of these components may vary with vegetable species, chemical nature, processing methods, storage time and conditions. Significant amounts of vitamin C, riboflavin and folate have been reported in many species of *Amaranthus* (Table 1). One hundred grams of these vegetables cooked without oil can contribute to 45% of daily vitamin A requirement (Mnkeni et al., 2007). For this reason and prevention of non communicable diseases, nutrition policies have therefore encouraged the consumption of diets containing more than 400 g/day of fresh vegetables and fruits especially in sub-Saharan Africa where many people are likely to suffer from vitamin A deficiency (Venneria et al., 2012).

Processing methods such as microwave-steaming and stir-frying with oil have been reported to offer greater retention of β-carotene in some vegetables than when boiled or stir-fried with water (Masrizal et al., 1997). On the other hand, eating cooked and pureed spinach leads to higher plasma total β-carotene concentrations, compared to raw consumption (Rock et al., 1998). This could be attributed to the heat destruction of enzymes that may be responsible for β-carotene degradation (Kala and Prakash, 2004). De Pee et al. (1995) reported that reduction in bioavailability of vitamin A from green leafy vegetables could be due to physical inaccessibility of carotenoids in plant tissues which may prevent the release of β-carotene from the matrix and competition for absorption with other carotenoids. Studies to determine the effects of traditional processing methods such as cooking and drying on the nutritional content of African indigenous leafy vegetables are therefore imperative as some of the methods could affect the nature and availability of important nutrients such as β-carotene.

Most ILVs also contain a significant amount of ascorbic acid (Table 1). It is however difficult to determine the contribution of ILVs to dietary vitamin C requirements since it is also greatly affected by cooking and processing methods including oxidative, enzymatic or photo degradation activities. Traditional methods of sun drying which do not involve blanching and sulphiting have been reported to cause ascorbic acid loss in okra, sweet pepper and tomatoes by 46.5%, 69.7% and 74%, respectively (Osunde and Makama, 2007). Furthermore, decreases of 19%, 61% and 100% have been reported in cooked amaranth, dried *Vernonia amygdalina* and dried *Adonsonia digitata* respectively (Uusiku et al., 2010). Low temperature storage of dehydrated vegetables may be employed as a better alternative preservative method since it reduces the degradation of vitamin C and browning (Negi and Roy, 2001). Steam blanching, followed by dehydration have been reported as the most effective preservation methods in retaining ascorbic acid (Uusiku et al., 2010).

Other vitamins

Appreciable amounts of vitamins D, E, K, thiamine, niacin, riboflavin, folate, pantothenic acid, pyridoxine and cyanocobalamin have been reported in many African leafy vegetables (Akubugwo et al., 2007a,b; Uusiku et al.,

Table 1. Macronutrient/vitamin-content of indigenous leafy vegetables consumed in the rural areas of South Africa.

Botanical name	Family	Macronutrient					Vitamin				Reference (s)
		H_2O	Carbs	Prot	Fibre	Fat	Vit. A	Vit. C	Ribof.	Folate	
Amaranthus cruentus	Amaranthaceae	83-9[a]	4-8[a]	4-6[a]	3[a]	0.2-0.6[a]	327[d]	46-126[d]	0.1-0.4[d]	64[d]	(Odhav et al., 2007; Uusiku et al., 2010)
A. hybrdus	Amaranthaceae	83[a]	6.09[a]	6[a]	2.81[a]	0.5[a]	327[d]	46-126[d]	0.1-0.4[d]	64[d]	(Odhav et al., 2007; Uusiku et al., 2010)
A. dubius	Amaranthaceae	85[a]	7.86[a]	4[a]	2.87[a]	0.2[a]	327[d]	46-126[d]	0.1-0.4[d]	64[d]	(Odhav et al., 2007; Uusiku et al., 2010)
A. spinosus	Amaranthaceae	83-9[a]	4-8[a]	4-6[a]	3[a]	0.2-0.6[a]	327[d]	46-126[d]	0.1-0.4[d]	64[d]	(Odhav et al., 2007; Uusiku et al., 2010)
A. thunbergii	Amaranthaceae	83-91[a]	4-8[a]	4-6[a]	3[a]	0.2-0.6[a]	327[d]	46-126[d]	0.1-0.4[d]	64[d]	(Odhav et al., 2007; Uusiku et al., 2010)
Cucurbita pepo	Cucurbitaceae	967.7[c]	26.23[c]	2.08[c]	3.72[c]	0.55[c]	194[d]	11[d]	0.1[d]	36[d]	(Kim et al., 2012)
C. moschata	Cucurbitaceae	942.31[c]	43.39[c]	3.05[c]	7.41[c]	0.89[c]	194[d]	11[d]	0.1[d]	36[d]	(Kim et al., 2012)
C. maxima	Cucurbitaceae	840.43[c]	133.53[c]	11.31[c]	10.88[c]	4.20[c]	194[d]	11[d]	0.1[d]	36[d]	(Kim et al., 2012)
Cucumis africanus	Cucurbitaceae	-	-	-	-	-	-	-	-	-	-
Cleome gynandra L.	Capparaceae	81.8-89.6[b]	4.4-6.4[b]	3.1-7.7[b]	1.3-1.4[b]	-	1200[d]	13-50[d]	0.1[d]	217[d]	(van der Walt et al., 2008; Uusiku et al., 2010; Mishra et al., 2011)
Cleome monophylla	Capparaceae	88[a]	3.40[a]	5[a]	2.14[a]	0.7[a]	1200[d]	13-50[d]	0.1[d]	346[d]	(Odhav et al., 2007; Uusiku et al., 2010)
Solanum nigrum	Solanaceae	83-90[a]	2[a]	3-5[a]	2-6[a]	0.6[a]	1070[d]	2[d]	0.3[d]	404[d]	(Uusiku et al., 2010)
Solanum retroflexum	Solanaceae	83-90[a]	2[a]	3-5[a]	2-6[a]	0.6[a]	1070[d]	2[d]	0.3[d]	404[d]	(Uusiku et al., 2010)
Chenopodium album	Chenopodiaceae	83[a]	8.34[a]	5[a]	1.92[a]	0.8[a]	917[d]	31[d]	0.3[d]	30[d]	(Odhav et al., 2007; Uusiku et al., 2010)
Portulaca oleracea	Portulacaceae	93[a]	2.65[a]	3[a]	1.21[a]	0.3[a]	-	-	-	-	(Odhav et al., 2007)
P. afra	Portulacaceae	-	-	-	-	-	-	-	-	-	-
Bidens pilosa	Asteraceae	85-88[a]	2[a]	3-5[a]	3-6[a]	0.4-0.6[a]	985-305[d]	23[d]	0.2[d]	351[d]	(Uusiku et al., 2010)
Corchorus olitorius	Tiliaceae	-	695[c]	162.6[c]	20.30[c]	17.2[c]	-	-	-	-	(Ndlovu and Afolayan, 2008)
Senna occidentalis	Fabaceae	77[a]	9.37[a]	7[a]	2.58[a]	2.2[a]	-	-	-	-	(Odhav et al., 2007)
Vigna unguiculata	Leguminosae	86[a]	2[a]	5[a]	4[a]	0.4[a]	99[d]	50[d]	0.2[d]	141[d]	(Uusiku et al., 2010)
Brassica rapa	Brassicaceae	92-94[a]	5-6[a]	1-2[a]	2-4[a]	0.1-0.3[a]	-	30-113[d]	0-0.2[d]	16[d]	(Uusiku et al., 2010)

H_2O, Water; Carbs, carbohydrates; Prot, proteins; Vit. A, vitamin A; Vit. C, vitamin C; Ribof, Riboflavin; [a], g/100 g of fresh weight; [b], % or mg/100g edible parts; [c], g/kg raw weight; [d], µg/100 g fresh weight; -, not determined.

2010; Erukainure et al., 2011). Various species of *Amaranthus*, *Cucurbita*, *Solanum*, *Brassica* and *Cleome* contain significant amounts of these vitamins (Table 1). Folate amounts of between 72 µg/100 g and 217 µg/100 g have been reported in some *Amaranthus* species including *Amaranthus hybridus* and *A*. *thunbergii*. (van der Walt et al., 2009). If consumed on a daily basis therefore, these vegetables could be an important source of dietary folate. Antioxidant and anti-inflammatory functions of folate and other components of ILVs are very important to improve the health of many South Africans at risk of cardiovascular diseases and also meet the high folate requirements of expectant mothers (van der Walt et al., 2008; 2009). In combination with tetrahydrobiopterin and insulin, folate has been reported to suppress superoxide anion generation and increase endothelial nitric oxide and prostacyclin production, both of which are potent platelet anti-aggregators and vasodilators (Lombardo and Chicco, 2006).

MINERAL COMPOSITION OF ILVS

Indigenous leafy vegetables are important sources of dietary minerals such as iron, zinc, calcium, magnesium, sodium, potassium and phosphorus and unlike vitamins; minerals are more stable to cooking and processing methods (Akubugwo et al., 2007a, b; Odhav et al., 2007; van der Walt et al., 2009). Their ratios, particularly sodium/potassium ratios are vital in the control of high blood pressure while calcium and phosphorus are important in the growth and maintenance of bones, teeth and muscles (Akubugwo et al., 2007a, b). Iron is an important element in the formation of haemoglobin and normal functioning of the central nervous system (Odhav et al., 2007). It is therefore very useful in the control of anaemia, especially in children and expectant mothers living in malaria endemic-regions of Africa (Akubugwo et al., 2007a,b; Uusiku et al., 2010). However, it occurs in the form of non haem iron and its absorption is influenced by factors such as the iron status of the individual, and several factors in the diet such as the presence of inhibitors (oxalates, phytate and fibre) (Kumari et al., 2004) and enhancers (ascorbic acid, β-carotene, fermentable carbohydrates and organic acids) (Uusiku et al., 2010). Just like iron, the absorption of zinc is also inhibited by phytates. Its deficiency may impair normal gastrointestinal and immune function (Uusiku et al., 2010).

Vegetable amaranth, *Solanum nigrum*, *Cleome gynandra* and other dark African leafy vegetables have been well documented as excellent sources of iron (Kumari et al., 2004; Faber et al., 2007; Maina and Mwangi, 2008; van der Walt et al., 2009). The mineral content of these vegetables and others as reported by different investigators are presented in Table 2, modified from Odhav et al. (2007) and Uusiku et al. (2010).

ANTIOXIDANT PROPERTIES OF ILVS AND THEIR ROLE IN HEALTH-MAINTENANCE

Generally, vegetables are good sources of roughages, providing an indigestible matrix which stimulates intestinal muscles and keep them in working order and also prevent constipation through their laxative effect (Seidu et al., 2012). Apart from nutritive value, *Amaranthus spinosus*, *A*. *hybridus*, *A*. *dubius*, *Cleome monophylla*, *Chenopodium album* and other ILVs when included in human diet are also known to play a role in reducing the incidence of oxidative stress-related diseases due to beneficial health functionality of their phenolic constituents (Akula and Odhav, 2008; Jimoh et al., 2011). These bioactive non-nutrient phytochemicals have the potential to reduce the risk of many degenerative human diseases and enhance the immune defence (Onyeka and Mwambekwe, 2007; van der Walt et al., 2009). They include flavonoids, hydrolysable and condensed tannins, coumarins, phenolic acids, stilbenes, lignans and lignins (Uusiku et al., 2010). Most of them are important free radical scavengers with higher *in vitro* antioxidant capacity than vitamins (Gardner et al., 2000; van der Walt et al., 2009). They retard or prevent deterioration, damage or destruction by oxidation (Bhuiyan et al., 2009). Some have the potential to reduce low density lipoprotein, which is the cholesterol involved in depositing fat in the arteries and prevents blood clotting which can reduce the risk for a heart attack or a stroke (Onyeka and Mwambekwe, 2007). Sulphur-containing components, some of which are found in ILVs are known to reduce cholesterol-production in the body thereby helping to keep the blood pressure down. As antioxidants, the phenolic constituents of ILVs protect cells from the damaging effects of free radicals arising from cellular redox reactions (Ebrahimzadeh et al., 2010).

Free radicals are unstable oxygen compounds with an unpaired electron in the atomic electron shell (for example O_2, OH, H_2O_2, HOCl, O_3). They are also known as reactive oxygen species (Gramza et al., 2005). Since all molecules tend to have complete electron pairs, the radicals react aggressively with other molecules, trapping electrons away from them (Gramza et al., 2005). They may not be harmful at low concentrations but at high concentrations, they generate oxidative stress, a deleterious process that can damage cell structures, including lipids, proteins and DNA (Ebrahimzadeh et al., 2010). If free radicals are not removed from the system, they may cause problems including many diseases such as cancer, heart disease, neuro-degenerative diseases and stroke and are responsible for aging (Bhuiyan et al., 2009). According to Lamien-Meda et al. (2008), the higher the polyphenolic constituent of the plant; the greater it's free radical-scavenging ability. Plant phenolic constituents may vary with species, geographical region, climate and age (Modi, 2007; Njume et al., 2011). Odhav et al. (2007) reported antioxidant activities of 96% for *Portulaca*

Table 2. Mineral content (mg/100g) of indigenous leafy vegetables consumed in the rural areas of South Africa (Odhav et al., 2007; Uusiku et al., 2010).

Plant name	Mineral					
	Fe	Ca	P	Na	Zn	Mg
Amaranthus cruentus	0.3-3.8[b]	253-425[b]	-	-	0.02-8.4[b]	105-224[b]
A. hybrdus	21[a]	2363[a]	604[a]	427[a]	18[a]	1317[a]
A. dubius	25[a]	1686[a]	487[a]	347[a]	56[a]	806[a]
A. spinosus	32[a]	3931[a]	629[a]	393[a]	1[a]	1156[a]
A. thunbergii	0.3-3.8[b]	253-425[b]	-	-	0.02-8.4[b]	105-224[b]
Cucurbita pepo	1.5[b]	39[b]	-	-	0.06-0.2[b]	38[b]
C. moschata	1.5[b]	39[b]	-	-	0.06-0.2[b]	38[b]
C. maxima	1.5[b]	39[b]	-	-	0.06-0.2[b]	38[b]
Cucumis africanus	-	-	-	-	-	-
Cleome gynandra	2.6-2.9[b]	31-288[b]	-	-	0.6-0.8[b]	44-76[b]
Cleome monophylla	24[a]	3203[a]	784[a]	25[a]	5[a]	371[a]
Solanum nigrum	85[a]	2067[a]	478[a]	431[a]	23[a]	277[a]
Chenopodium album	13[a]	1490[a]	797[a]	683[a]	109[a]	1239[a]
Portulaca oleracea	42[a]	1361[a]	333[a]	148[a]	34[a]	1037[a]
P. afra	-	-	-	-	-	-
Bidens pilosa	17[a]	1354[a]	504[a]	290[a]	22[a]	658[a]
Corchorus olitorius	2.0[b]	-	-	-	0.05[b]	-
Senna occidentalis	11[a]	2230[a]	417[a]	347[a]	9[a]	854[a]
Vigna unguiculata	0.3-3.0[b]	188[b]	-	-	0.23[b]	60[b]
Brassica rapa	0.5-3.5[b]	27-31[b]	-	-	0.9-1.3[b]	13[b]

Fe, Iron; Ca, calcium; P, phosphorus; Na, Sodium; Zn, zinc; Mg, magnesium; [a], mg/100 g dry weight; [b], mg/100 g fresh weight; -, not determined.

oleracea and *Justicia flava* and 92% for *Solanum nigrum* while Stangeland et al. (2009) reported antioxidant activities of 1.56 mmolTE/100 g, 1.0 mmolTE/100 g and 0.87 mmolTE/100 g for *Cleome gynandra*, A*maranthus* species and *Solanum macrocarpon* respectively.

Due to the generally low level of crude fat in many locally consumed indigenous vegetable leaves and their high levels of total unsaturated fatty acid (van der Walt et al., 2008), consumption in large amounts would be beneficial to individuals suffering from overweight or obesity, and this would constitute a good dietary habit (Erukainure et al., 2011). Ascorbic acid found in most ILVs is a free radical scavenger and in addition, is able to regenerate other antioxidants such as tocopheroxyl and the carotene radical cation from their radical species (Uusiku et al., 2010). It is important to note that some of the components classified as antioxidants, for example, tannins reduce the availability of certain nutrients such as proteins and starch by forming complexes with them or the enzymes required for their metabolism. Tannins alongside phenolic acids and flavonoids also reduce iron availability and interfere with protein absorption (Uusiku et al., 2010).

CONCLUSION

Consumption of ILVs could offer significant health-protection benefits given that some of these crops are functional foods with health-promoting and immune-strengthening properties. Considering their potential nutritional value, ILVs could contribute in a major way to the food security and balanced diets of rural households in South Africa and different parts of the world. Identifying ILVs of high nutrient content could be a major step in addressing South Africa's food security problems. There is a need to create market awareness for ILVs considering that they are fairly easy to cultivate, resistant to pest and disease and produce very stable yields even under difficult climatic conditions. In order to avert the loss of micronutrients by traditional processing methods, we advocate the use of shade drying to reduce photo degradation, thin slicing to reduce drying time and use of pre-drying treatment such as blanching or sulphating to reduce enzyme activities and loss of vitamins.

Conflict of Interests

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

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REFERENCES

Adebooye OC, Opabode JT (2004). Status of conservation of the indigenous leaf vegetables and fruits of Africa. Afr. J. Biotechnol. 3: 700-705.

Akubugwo IE, Obasi AN, Ginika SC (2007a). Nutritional potential of the leaves and seeds of black nightshade-*Solanum nigrum* L. var virginicum from Afikpo-Nigeria. Pak. J. Nutr. 6: 323-326.

Akubugwo IE, Obasi NA, Chinyere GC, Ugbogu AE (2007b). Nutritional and chemical value of *Amaranthus hybridus* L. leaves from Afikpo, Nigeria. Afr. J. Biotechnol. 6: 2833-2839.

Akula US, Odhav B (2008). In vitro 5-lipoxygenase inhibition of polyphenolic antioxidants from undomesticated plants of South Africa. J. Med. Plants Res. 2: 207-212.

Alvarez-Jubete L, Arendt LK, Gallagher E (2010). Nutritive value of pseudocereals and their increasing use as functional gluten-free ingredients. Trends Food Sci. Technol. 21:106-113.

Aregheore EM (2012). Nutritive value and inherent anti-nutritive factors in four indigenous edible leafy vegetables in human nutrition in Nigeria: a review. J. Food Resour. Sci. 1: 1-14.

Aremu MO, Olaofe O, Akintayo TEA (2006). comparative study on the chemical and amino acid composition of some Nigerian under-utilized Legume flours. Pak. J. Nutr. 5: 34-38.

Arthur GD, Naidoo KK, Coopoosamy RM (2012). *Bidens pilosa* L. Agricultural and pharmaceutical importance. J. Med. Plants Res. 6: 3282-3287.

Bartolome AP, Villasenor IM, Yang WC (2013). *Bidens pilosa* L (Asteraceae): botanical properties, traditional uses, phytochemistry, and pharmacology. Evidence-Based Comp. Alt. Med. 2013:1-51.

Bhuiyan MAR, Hoque MZ, Hossain SJ (2009). Free radical scavenging activities of *Zizyphus mauritiana*. World J. Agric. Sci. 5: 318-322.

Black R (2003). Micronutrient deficiency – an underlying cause of morbidity and mortality. Bull. World Health Org. 81: 79.

Chung SW, Madulid DA, Hsu TC (2008). *Portulaca psammotropha* Hance (Portulacaceae), a neglected species in the flora of Taiwan and Philippines. Tawainia 53: 90-95.

de Pee S, West CE, Muhilal L, Daryadi D, Hautvast JGAJ (1995). Lack of improvement in vitamin A status with increased consumption of dark green leafy vegetables. Lancet 346: 75-81.

Dweck AC (2001). Purslane (*Portulaca oleracea*)-the global panacea. Per. Care Mag. 2: 7-15.

Ebrahimzadeh MA, Nabavi SM, Nabavi SF, Bahramian F, Bekhradnia AR (2010). Antioxidant and free radical scavenging activity of *H. officinalis* L. var angustifolius, *V. odorata*, *B. hyrcana* and *C. speciosum*. Pak. J. Pharm. Sci. 23: 29-34.

Edmonds JM, Cheweya JA (1997). Promoting the conservation and use of underutilized and neglected crops. Available online http://www.underutilized species.org/documents/ Publications/ black_nightshades.pdf . Accessed 05/02/2013

Erukainure OL, Oke OV, Ajiboye AJ, Okafor OY (2011). Nutritional qualities and phytochemical constituents of *Clerodendrum volubile*, a tropical non-conventional vegetable. Int. Food Res. J. 18:1393-1399.

Faber M, van Jaarsveld PJ, Laubscher R (2007). The contribution of dark-green leafy vegetables to total micronutrient intake of two- to five-year-old children in a rural setting. Water SA 33 (3): 407-412.

Faber M, Witten C, Drimie S (2011). Community-based agricultural interventions in the context of food and nutrition security in South Africa. S. Afr. J. Clin. Nutr. 24: 21-30.

Gardner PT, White TAC, Mcphail DB, Duthie GG (2000). The relative contributions of vitamin C, carotenoid and phenolics to the antioxidant potential of fruit juices. Food Chem. 68: 471-474.

Gqaza MB, Njume C, Goduka IN, Grace G (2013). Nutritional assessment of *Chenopodium album* L. (Imbikicane) young shoots and mature plant-leaves consumed in the Eastern Cape Province of South Africa. Int. Conf. Nutr. Food Sci. DOI: 10.7763/IPCBEE.

Gramza A, Pawlak-Lemanska K, Korczak J, Wasowicz E, Rudzinska M (2005). Tea extracts as free radical scavengers. Pol. J. Environ. Stud. 14: 861-867.

Hassan LG, Umar KJ (2006). Nutritional value of Balsam Apple (*Momordica balsamina* L.) leaves. Pak. J. Nutr. 5: 522-529.

He F, Nowson CA, MacGregor GA (2006). Fruit and vegetable consumption and stroke: meta-analysis of cohort studies. Lancet 367: 320-326.

Jimoh FO, Adedapo AA, Afolayan AJ (2011). Comparison of the nutritive value, antioxidant and antibacterial activities of *Sonchus asper* and *Sonchus oleraceus*. Rec. Nat. Prod. 5: 29-42.

Kala A, Prakash J (2004). Nutrient composition and sensory profile of differently cooked green leafy vegetables. Int. J. Food Prop. 7: 659-669.

Kim MY, Kim EJ, Kim YN, Choi C, Lee BH (2012). Comparisons of the chemical compositions and nutritive values of various pumpkin (Cucurbitaceae) species and parts. Nutr. Res. Pract. 6: 21-27.

Kleynhans R, Myeza PN, Laurie SM, Visser A, van Rensburg JWS, Adebola PO (2013). Collection, maintenance and utilization of plant genetic resources at Agricultural Research Council (ARC)-Roodeplaat Vopi, South Africa. Acta Hort. (ISHS) 1007:993-998.

Kumari M, Gupta S, Lakshmi J, Prakash J (2004). Iron bioavailability in green leafy vegetables cooked in different utensils. Food Chem. 86: 217-222.

Kwenin WKJ, Wolli M, Dzomeku BM (2011). Assessing the nutritional value of some Affrican indigenous green leafy vegetables in Ghana. J. Anim. Plant Sci. 10: 1300-1305.

Labadarios D (2005). National food consumption survey-fortification baseline (NFCS-FB). Department of Health, Pretoria, South Africa.

Lamien-Meda A, Lamien CE, Compaore MMY, Meda RNT, Kiendrebeogo M, Zeba B, Millogo JF, Nacoulma OG (2008). Polyphenol content and antioxidant activity of fourteen wild edible fruits from Burkina Faso. Molecules 13: 581-594.

Lewu FB, Mavengahama S (2010). Wild vegetables in Northern KwaZulu Natal, South Africa: current status of production and research needs. Sci. Res. Essays 5: 3044-3048.

Lombardo YB, Chicco AG (2006). Effects of dietary polyunsaturated n-3 fatty acids on dyslipidemia and insulin resistance in rodents and humans. A review. J. Nutr. Biochem. 17: 1-13.

Maanda MQ, Bhat RB (2010). Wild vegetable by Vhavenda in the Venda region of Limpopo province, South Africa. Int. J. Exp. Bot. 79: 189-194.

Maina S, Mwangi M (2008). Vegetables in East Africa. Elewa Pub. 1:1-11.

Makobo ND, Shoko MD, Mtaita TA (2010). Nutrient content of amaranth (*Amaranthus cruentus* L.) under different processing and preservation methods. World J. Agric. Sci. 6: 639-643.

Masrizal MA, Giraud DW, Driskell JA (1997). Retention of vitamin C, iron and bcarotene in vegetables prepared using different cooking methods. J. Food Qual. 20: 403-418.

Matenge STP, van der Merwe D, de Beer H, Bosman MJC, Kruger A (2012). Consumers' beliefs on indigenous and traditional foods and acceptance of products made with cow pea leaves. Afr. J. Agric. Res. 7: 2243-2254.

Mishra SS, Moharana SK, Dash MR (2011). Review on *Cleome gynandra*. Int. J. Res. Pharm. Chem. 1: 681-689.

Misra S, Maikhuri RK, Cala CP, Rao KS, Saxena, KG (2008). Wild leafy vegetables: a study of their subsistence dietetic support to the inhabitants of Nanda Devi biosphere reserve. Indian J. Ethnobiol. Ethnomed. 4:15.

Mlakar SG, Turinek M, Jacop M, Bavec M, Bavec F (2010). Grain Amaranth as an alternative and perspective crop in temperate climate. J. Geogr. 5: 135-145.

Mnkeni AP, Masika P, Maphaha M (2007). Nutritional quality of vegetables and seeds from different accessions of *Amaranthus* in South Africa. Water S. Afr. 33: 377-380.

Modi AT (2007). Growth temperature and plant age influence on nutritional quality of *Amaranthus* leaves and seed germination capacity. Water S. Afr. 33: 369-376.

Ndlovu J, Afolayan AJ (2008). Nutritional analysis of South African wild vegetables *Corchorus olitorius* L. Asian J. Plant Sci. 7: 615-618.

Negi, PS, Roy SK (2001). Effect of drying conditions on quality of green leaves during long term storage. Food Res. Int. 34: 283-287.

Njume C, Afolayan AJ, Green E, Ndip RN (2011). Volatile compounds in the stem bark of *Sclerocarya birrea* (Anacardiaceae) possess

antimicrobial activity against drug-resistant strains of *Helicobacter pylori*. Int. J. Antimicrob. Agents 38: 319-324.
Nnamani, CV, Oselebe HO, Agbatutu A (2009). Assessment of nutritional value of three underutilized indigenous leafy vegetables of Ebony State, Nigeria. Afr. J. Biotechnol. 8: 2321-2324.
Nojilana B, Norman R, Bradshaw D, van Stuijvenberg ME, Dhansay MA, Labadarios D (2007). Estimating the burden of disease attributable to vitamin A deficiency in South Africa in 2000. S. Afr. Med. J. 97: 748-753.
Nyuar KB, Ghebremeskel K, Crawford MA (2012). Sudanese women and neonates' vitamin A status. Nutr. Health 21: 45-55.
Odhav B, Beekrum S, Akula US, Baijnath H (2007). Preliminary assessment of nutritional value of traditional leafy vegetables in KwaZulu-Natal, South Africa. J. Food Compost. Anal. 20: 430-435.
Ojieh G, Oluba O, Ogunlowo Y, Adebisi K, Eigdangbe G, Orole R (2007). Compositional studies of *Citrullus lanatus* (Egusi melon) seed. Internet J. Nutr. Wellness 6(1):19-26.
Onyeka EU, Nwambekwe IO (2007). Phytochemical profile of some green leafy vegetables in South East, Nigeria. Niger. Food J. 25: 67-76.
Osunde ZD, Makama M (2007). Assessment of changes in nutritional values of locally sun-dried vegetables. Assump. Univ. J. Technol. 10: 248-253.
Puoane T, Steyn K, Bradshaw D, Laubscher R, Fourie J, Lambert V, Mbananga N (2002). Obesity in South Africa: The South African demographic and health survey. Obes. Res. 10: 1038-1048.
Rock CL, Lovalvo JL, Emenhiser C, Ruffin MT, Flatt SW, Schwartz SI (1998). Bioavailability of β-carotene is lower in raw than in processed carrots and spinach in women. J. Nutr. 128: 913-916.
Sasson A (2012). Food security for Africa: an urgent global challenge. Agric. Food Sec. 1:2.
Seidu JM, Bobobee EYH, Kwenin WKJ, Frimpong R, Kubje SD, Tevor WJ, Mahama AA (2012). Preservation of indigenous vegetables by solar drying. ARPN J. Agric. Biol. Sci. 7: 407-416.
Shackleton CM (2003). The prevalence of use and value of wild edible herbs in South Africa. S. Afr. J. Sci. 99: 23-25.
Singh KP, Dwevedi AK, Dhakre G (2011). Evaluation of antibacterial activity of *Chenopodium album* L. Int. J. Appl. Biol. Pharm. Technol. 2: 398-401.
Silva FL, Fischer DCH, Tavares JF, Silva MS, De Athayde-Filho PF, Barbosa-Filho JM (2011). Compilation of secondary metabolites from *Bidens pilosa* L. Molecules 16(2): 1070-1102.
Stangeland T, Remberg SF, Lye KA (2009). Total antioxidant activity in 35 Ugandan fruits and vegetables. Food Chem. 113: 85-91.
Sundriyal M, Sundriyal RC (2001). Wild edible plants of the Sikkim Himalaya: Nutritive values of selected species. Econ. Bot. 55: 377-390.
Umar KJ, Hassan LG, Dangoggo SM, Maigandi SA, Sani NA (2011). Nutritional and anti-nutritional profile of spiny amaranth (*Amaranthus viridis* Linn). Studia Universitatis Vasile Goldis Seria Stiintele Vietii 21(4): 727-737.
UNICEF, 2006. The State of the World's Children 2007 – Executive Summary. Women and Children, the Double Dividend of Gender Equality. New York, USA
Uusiku NP, Oelofse A, Duodu KG, Bester MG, Faber M (2010). Nutritional value of leafy vegetables of Sub-Saharan Africa and their potential contribution to human health: A review. J. Food Compost. Anal. 23: 499-509.
van Averbeke W, Tshikalange TE, Juma KA (2007). The commodity systems of *Brassica rapa* L. susbsp. *Chinensis* and *Solanum retroflexum* Dun. In Vhembe, Limpopo province, South Africa. Water S. Afr. 33: 349-354.
van der Walt AM, Ibrahim MI, Benzuidenhout CC, Loots DT (2008). Linolenic acid and folate in wild-growing African dark leafy vegetables (Morogo). Pub. Health Nutr. 12:525-530.
van der Walt AM, Loots DT, Ibrahim MIM, Benzuidenhout CC (2009). Minerals, trace elements and antioxidant phytochemicals in wild African dark-green leafy vegetables (morogo). S. Afr. J. Sci. 105: 444-448.
van Jaarsveld P, Faber M, van Heerden I, Wenhold F, van Rensburg WJ, van Averbeke W (2014). Nutrient content of eight African leafy vegetables and their potential contribution to dietary reference intakes. J. Food Compost. Anal. 33:77-84.
van Rensburg JWS, Venter, SL, Netschluvhi TR, Heever E, Vorster HJ, de Ronde JA (2004). Role of indigenous leafy vegetables in combating hunger and malnutrition. S. Afr. J. Bot. 70: 52-59.
van Rensburg WSJ, van Averbeke W, Slabbert R, Faber M, van Jaarsveld P, van Heerden I, Wenhold F, Oelofse A (2007). African Leafy Vegetables in South Africa. Water S. Afr. 33: 317-326.
Venneria E, Marinelli L, Intorre F, Foddai MS, Aurigemma C, Durazzo A, Maiani G, de Giusti M (2012). Effect of harvest time and minimal processing on nutritional and microbiological quality of three leaf crops. J. Agric. Biodivers. Res. 1: 11-17.
Voster HJ, van Rensburg WSJ (2005). Traditional vegetables as a source of food in South Africa: Some experiences. Afr. Crop Sci. Conf. Proc. 7: 669-671.
WHO (2009). Global Prevalence of Vitamin A Deficiency in Populations at Risk 1995–2005: WHO Global Database of Vitamin A Deficiency. Geneva, Switzerland.
Yadav N, Vasudeva N, Singh S, Sharma SK (2007). Medicinal properties of genus *Chenopodium* Linn. Nat. Prod. Rad. 6: 131-134.

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Random amplified polymorphic DNA (RAPD) based assessment of genetic relationships among some Zimbabwean sorghum landraces with different seed proanthocyanidin levels

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Knowledge of genetic distances between genotypes is important for efficient organization and conservation of plant genetic resources for crop improvement programs. In this study genetic distances between genotype pairs (complements of Jaccard's similarity coefficient) were estimated from Random Amplified Polymorphic DNA (RAPD) data collected from 48 Zimbabwean sorghum landraces. These varieties showed variation in their seed proanthocyanidin (PAs) levels with 16 and 29 of them having detectable and non-detectable PA levels respectively. RAPDs revealed considerable genetic variation between the varieties used and 2.7 polymorphisms per primer were obtained. Ninety nine polymorphic RAPD bands were used to calculate genetic distances and the mean genetic distance between the genotypes was 0.494 (± 0.113) with a range of 0.051 to 0.761. A multidimensional scaling (MDS) plot of the distance matrix revealed two distinct clusters of cultivated and wild sorghums. No clustering of genotypes according to their seed proanthocyanidin levels was revealed by MDS analysis; also the mean genetic distances of genotypes in the low, medium and high PA categories were not different from each other and none of them was significantly different from the mean genetic distances between all the groups. The RAPD markers used in the present study could not distinguish between sorghums with different PA levels in their seeds; however, the protocol established could be useful in further analysis of this trait in near isogenic lines.

Key words: Genetic distances, multidimensional scaling, proanthocyanidins, Random Amplified Polymorphic DNA (RAPD), *Sorghum bicolor,* Zimbabwe

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) is a traditional cereal crop in Zimbabwe and it ranks fourth in production after maize, wheat, and pearl millet (FAO, 2006). Sorghum utilization is generally influenced by the presence

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of polyphenolic compounds that are produced in large quantities in grain and vegetative tissues of many cultivars (Waniska, 2000). The polyphenolic compounds of sorghum such as the proanthocyanidins (PAs), also known as condensed tannins, have protein binding properties, which tend to reduce the nutritional quality of sorghum based diets in both livestock and humans. However, despite their nutritional side effects, sorghum polyphenols have been implicated in defense against competitors, herbivores and pathogens (Winkel-Shirley, 2001). There is need to develop high yielding sorghum cultivars with desirable levels and types of polyphenolic compounds that will improve the nutritional qualities of the crop without compromising the positive agronomic traits conferred by these compounds.

Future sorghum improvement programmes have increased the utilization of local germplasm resources. There are indications that communal sorghum farmers are selecting some local landraces for traits such as drought and pest tolerance, disease resistance, early maturity, palatability and storability among others (Nagaraj et al., 2013). The availability of high yielding sorghum cultivars with traits desired by farmers, is likely to result in increased sorghum production and thus improved food security in the semi-arid regions.

Zimbabwe is one of the few African countries with a rich and varied gene pool of sorghum landraces that has been selected and built-up over centuries (Chakauya et al., 2006). If this germplasm is to be widely utilized in sorghum breeding programmes, there is need for detailed understanding of its genetic diversity. This genetic diversity can be evaluated by one of several means, most of which enable the estimation of genetic distances between the landraces (Chakauya et al., 2006; Abdel-Fatah et al., 2013; Ng'uni et al., 2011). Information on the degree to which lines or populations are genetically related can help breeders in making plans for genetic crosses, in assigning available breeding materials to specific heterotic groups, and in the identification of individual varieties with reference to plant varietal purity and its maintenance (Mohammadi and Prasanna, 2003). Furthermore, knowing the level of genetic variation in a germplasm collection can facilitate more efficient sampling of genotypes for particular needs and identifying lines that should be kept to preserve maximum genetic diversity in germplasm banks, thereby facilitating efficient handling of germplasm resources (Bretting and Widrlechner, 1995).

There are different genetic markers such as morphological traits, protein and DNA markers that can be used in genetic diversity studies. Random amplified DNAs (RAPDs) were used in this study because they are relatively cheap and easy to perform; they require small amounts of DNA material detect relatively small amounts of genetic variation and enable inexpensive generation of data that can be subjected to different statistical manipulations.

RAPD based assessment of genetic similarities in plants usually employs either one of the three commonly used similarity coefficients (Dudley 1994), which are the simple matching coefficient (Sneath and Sokal, 1973), Jaccard's coefficient (Gower, 1972) and Nei and Li's coefficient (Nei and Li, 1979). All these similarity coefficients are non-negative and have an upper limit of unity. In such cases where the similarity measure is bound by zero and unity there is always a dissimilarity, this dissimilarity is the genetic distance between the two genotypes *i* and *j.* Just like similarity, the dissimilarity is symmetric and non-negative. Naturally, an organism has maximal similarity to itself, thus S_{ii}=1 and GD_{ij}=0 will mean no genetic difference, while GD_{ij}=1 signifies complete difference between the two genotypes (Everitt, 1993; Nienhuis et al., 1994).

The genetic distances derived from heritable characteristics (genetic markers) define the phenetic patterns of a population (Abbott et al., 1985). Usually discontinuities exist in such patterns, resulting in groupings with different ranges of variation within groups and varying degrees of differences between them. It is important for the investigator to visualise these groupings because a lot of information for decision-making can be deduced from them. Thus, the third step in a genetic distance estimation study is to transform, by statistical methods, the genetic distance matrix into a diagrammatic form (clustering), from which the phenetic groupings can be easily identified. In molecular marker studies, dendogram construction and ordination techniques are the commonly used clustering techniques. Principal component analysis (PCA), principal co-ordinate analysis and non-metric multi-dimensional scaling (MDS) are the most commonly used ordination techniques. In this study MDS was used.

Our objective in this study was to use the RAPD technique to estimate genetic distances between some Zimbabwean sorghum landraces and to identify RAPD markers that can be used to discriminate between cultivars with different levels of proanthocyanidins in their seeds.

MATERIALS AND METHODS

Plant material

Forty-eight sorghum varieties collected from different parts of Zimbabwe were used in this study. These included 37 randomly sampled landraces, cultivated by rural farmers, 5 commercial cultivars, 3 breeder's experimental lines and 3 wild sorghums (*Sorghum arundinaceum* Desv) (Table.1).

Determination of proanthocyanidin (PAs) levels in sorghum seeds.

Forty five sorghum varieties in this study had their seeds tested for the presence of soluble and insoluble proanthocyanidins (PAs) using the butanol-HCl method described by Bate-Smith (1975). The three wild genotypes were not assayed for tannins because the quantity of seeds available was not adequate for the assay.

DNA extraction and quantification

Seeds of all the varieties were germinated in the greenhouse. Fresh leaf tissue was harvested from 7-day-old seedlings, per

Table 1. Sorghum cultivars used in the study, their common names, areas of origin and seed proanthocyanidin levels.

Genotype	Common Name	Area of Origin	PA Status	
			A550 nm*	Classification
Commercial cultivars				
SV1	SV1	Harare	0.043	Low
SV2	SV2	Harare	0.039	Low
ZWSH1	ZWSH1	Harare	0.005	Low
Experimental lines				
NL700	NL700	Harare	0.004	Low
NL829	NL829	Harare	0.026	Low
DC75m	DC75m	Harare	0.032	Low
DW	Dwarf Wonder	Harare	0.238	Medium
Wild Lines				
W9	Wild sorghum	Harare	U	Unknown
W10	Wild sorghum	Harare	U	Unknown
W15	Wild sorghum	Harare	U	Unknown
Local landraces				
PL01	Isitishi	Matopo	0.004	Low
PL02	Ntelwane	Matopo	0.031	Low
PL04	Katandanzara	Mutoko	0.007	Low
PL05	Ludende	Gwanda	0.002	Low
PL08	Iganu	Matopo	0.014	Low
PL09	Tswetha	Matopo	0.024	Low
PL10	Muchaina	Chipinge	0.005	Low
PL12	Rudende	Mutoko	0.003	Low
PL14	Brown tsweta	Gwanda	0.031	Low
PL15		Harare	0.020	Low
16Ga		Magaisa	0.003	Low
68Db	Sikhothama	Tsholotsho	0.029	Low
102K	Murara	St Joseph	0.041	Low
107D	Chifumbata	St Joseph	0.007	Low
370	Imfe/ipwa	Zaka	0.007	Low
499	Mugonellengo	Gata Camp	0.015	Low
581		Mt Selinda	0.009	Low
646		Mt Selinda	0.021	Low
653		Mt Selinda	0.005	Low
310		Zaka	0.004	Low
671		Zhombe	0.004	Low
686		Zhombe	0.005	Low
877	Imfe/ipwa	Manyoni	0.008	Low
886	Imfe/ipwa	Manyoni	0.557	Medium
4D		Chivi	0.426	Medium
7B	Chibedlani	Gutu	0.473	Medium
34K	Gangara	Malipati	0.138	Medium
44B		Lundi	0.292	Medium
71C	Tsveta	Shurugwi	0.389	Medium
304	Imfe/ipwa	Turgwana	0.417	Medium
304a	Imfe/ipwa	Turgwana	0.417	Medium
21G	Mugore	Kyle	0.639	High
37Ce	Chititshi	Malipati	0.958	High
PL03	Red Swazi	Chipinge	0.722	High
PL06	Chibonda	Mutoko	0.743	High
42K	Gangara	Lundi	0.880	High
We2		Harare	0.622	High
10B3c		Gutu	0.949	High

*Absorbance at 550 nm.

variety and 0.75 g of the pooled leaf tissue was used for DNA extraction. The DNA extraction buffer was modified from Jhingan (1992), with potassium ethyl xanthogenate (PEX), (Fluka Chemical Corp.,USA) replacing sodium ethyl xanthogenate. The weighed leaf tissue was ground to a fine pulp in 50 µL of PEX extraction buffer [200 mM Tris buffer (pH 7.5), 1.4 M NaCl, 600 mM PEX, 100 mM EDTA (pH 8.0)] in 1.5 ml micro centrifuge tubes using plastic grinding rods. An additional 450 µL PEX extraction buffer was added, the tubes were vortexed briefly and incubated in a water bath at 65°C for 1 h. Thereafter, the samples were centrifuged at 10 000 revolutions per minute (RPM) in a microcentrifuge for 10 min. Supernatants were transferred to clean microcentrifuge tubes containing 1000 µL of a 6:1 mixture of absolute ethanol and 7.5 M ammonium acetate to precipitate the nucleic acids at room temperature. After 30 min of precipitation, the nucleic acids were collected by centrifugation at 3000 RPM. The pellets were re-suspended in 300 µL of TE buffer (1 mM Tris pH 7.5; 0.1 mM EDTA pH 8.0) containing 100 µg/ml RNase A and incubated in a water bath at 37°C for 1 h. Any remaining tissue debris was pelleted from the suspension by centrifugation at 14 000 RPM for 1 min and the supernatants transferred to clean microcentrifuge tubes. To the supernatant, 1200 µL of a 10:1 mixture of absolute ethanol and 3 M sodium acetate was added to precipitate the DNA at room temperature for 30 min. The DNA precipitate was pelleted by centrifugation at 3000 RPM for 5 min. The pelleted DNA was washed by gentle vortexing in 70% ethanol followed by centrifugation at 14 000 RPM for 15 min to collect the clean pellet. The washed DNA pellet was air-dried and finally dissolved in 75 µL of TE buffer (pH 7.5). The DNA in each sample was quantified by using a DNA fluorometer (Hoeffer Scientific Instruments,USA) and diluted in TE-tartrazine (TE buffer with 200 mM tartrazine) to a working concentration of 4 ng/µL.

RAPD Procedures and Primer Screening

All RAPD reactions were done in a total volume of 10 µL. Each reaction was carried out in RAPDs buffer [50 mM Tris buffer (pH 8.5), 20 mM KCl, 3.5 mM $MgCl_2$, 0.05% (w/v) bovine serum albumin, 0.01% xylene cyanol and 1.25 % (w/v) Ficoll 400] on 20 ng of DNA, 1 unit of *Taq* DNA polymerase enzyme (Promega, USA), 1 µM of random decamer (Operon technologies, Alameda, California, USA and University of British Columbia, Canada) and 0.2 mM of each dNTP (Skroch and Nienhuis, 1995). Eight varieties representing four different seed PA groups (low, medium, high and unknown) were evaluated for genetic polymorphisms using 70 randomly selected RAPD primers. The PCR products were electrophoresed on 1.5% agarose gels, stained with Ethidium Bromide and photographed over UV light onto Polaroid 667 film. The gel pictures were then used to identify the primers which produced clear polymorphic bands. On this basis, 40 primers were selected for use in the main RAPD study for estimating genetic distances among the 48 sorghum varieties.

Thermal cycling conditions

All RAPD reactions were performed in thin walled 96-well plates in an MJ PC100 thermocycler (MJ Research, Water Town, MA, USA). A total of 39 cycles were performed, in the first cycle the temperature settings were: 91°C for denaturation for 60 s, 42°C for annealing for 15 s followed by elongation at 72°C for 70 s. The subsequent 38 cycles had denaturation time set at 15 s, annealing at 15 s and elongation at 70 s with temperatures similar to first cycle for each of the three PCR steps.

RAPD band scoring

Like all dominant molecular marker techniques RAPDs generate binary data, thus when comparing two genotypes *i* and *j* using this kind of data there are four possible outcomes: [1,1], [0,1], [1,0] or [0,0], (1= presence and 0= absence of a band (genetic marker) in genotype *i* and *j* respectively). Polymorphic bands were scored from the gel photographs. Monomorphic bands, which were the majority of bands seen on the sorghum RAPD gels, were not scored. Two criteria were used in scoring bands: firstly, the band had to stain strongly; secondly, there had to be an unambiguous difference between the allelic states of the band being scored, that is, presence or absence of a band. Each polymorphic band was treated as a unit character, and each variety was scored for the presence or absence of a band, scored 1 or 0, respectively.

Statistical analyses

The scored bands data were used to calculate genetic distances using the Jaccard's similarity coefficient (J_{ij}) (Gower, 1972):

$$J_{ij} = \frac{N_{(1,1)}}{N_{(1,1)}+N_{(1,0)}+N_{(0,1)}} \quad (1)$$

where $N_{(1,1)}$, $N_{(1,0)}$ and $N_{(0,1)}$ is the number of times the cultivars i and j both have a particular band, i has a band while j does not and j has a band while i does not, respectively. This similarity coefficient is non-negative and has an upper limit of unity. In such a case where the similarity measure is bound by zero and unity, there is always a dissimilarity, which is the genetic distance (GD_{ij}) between two genotypes *i* and *j*. Just like similarity the dissimilarity is symmetric and non-negative.

$$GD_{ij} = 1 - J_{ij} \quad (2)$$

Naturally, an organism has maximal similarity to itself, thus $S_{ii} = 1$ and $GD_{ij} = 0$ will mean no genetic difference, while $GD_{ij} = 1$ signifies complete difference between the two genotypes (Everitt, 1993; Nienhuis et al., 1994). Genetic distances between all the 1128 possible genotype pairs [n(n-1)/2, where n is the number of genotypes in the study] from the 48 accessions were calculated using the correlation procedure of the statistical programme, SYSTAT 5.2 (Wilkinson, 1992). This produced a 48 × 48 genetic distance matrix. For the purposes of visualizing the genetic relationships between the cultivars with different seed proanthocyanidin levels, the cultivars were classified into four groups based on the butanol HCl assay for PAs. The four groups were; high, medium and low (with absorbance greater than 0.5, between 0.140 and 0.5, and less or equal to 0.05, respectively) and unknown in the case of wild lines whose PA levels were not determined (Table 1). The genetic distance matrix was converted to two-dimensional coordinates using the multidimensional scaling (MDS) procedure in SYSTAT 5.2. The objective of MDS is to estimate the coordinates of a set of genotypes in a space of specified dimensionality from data measuring the relationships between pairs of genotypes (SAS Institute, 1990; Schiffman et al., 1981). The coordinates are supposed to represent the information from the genetic matrix so that there is maximum correspondence between the observed proximities and inter-point distances (Everitt, 1993).

Thus the larger the calculated genetic distance between two individuals the further apart should the points representing them on the plot. To determine if a subset of one or more RAPD bands could be selected that would allow classification of the cultivated sorghum genotypes into three proanthocyanidin groups (high, medium and low), an additional analysis was performed. This involved ranking the RAPD bands according to their ability to separate the cultivars into the three groups, thus maximizing variances of band frequencies among groups; this was done by calculating individual

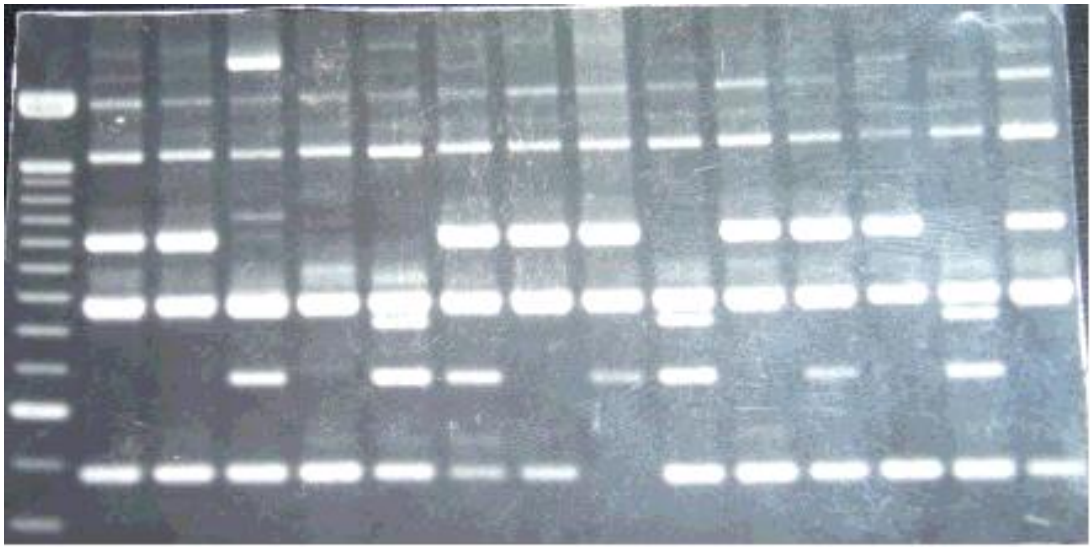

Figure 1. RAPD banding patterns with primer OPAS-14 in 15 sorghum cultivars: 10B3c, 304, 304a, 34K, PL14, 4D, 646, 877, DW, 42K 7B, PL9, DC75, 16Ga and PL10 respectively. The first lane is a 100 bp molecular weight marker.

band frequencies in all the groups. In this case the frequency of a band is the proportion of genotypes in a particular group having the band, relative to all evaluated genotypes. The bands were then ranked by variance of band frequencies across groups. The best 15 bands were selected and used to calculate new genetic distances and the genetic distance matrix was used in MDS analysis as described above. This analysis did not give a clear distinction between the three groups of cultivated sorghums. The classification was then changed to include only two PA groups; those with detectable PA levels (high) and those without (low). Band frequencies among these two groups were calculated and 15 bands with the greatest differences in amplification frequencies between the two groups were used to compute genetic distances and MDS analysis was performed on the resultant distance matrix.

RESULTS

Variation in seed PA content

The sorghum landraces and commercial cultivars used in this study showed significant variability in their seed proanthocyanidin levels. Of the 45 genotypes assayed for PAs using the butanol-HCl assay, 16 (36%) had detectable PA levels while 29 (64%) did not have detectable PA levels (Table 1).

Degree of genetic polymorphisms in sorghums as revealed by RAPDs

Of the 70 primers screened for their ability to detect polymorphisms in sorghum, only 5 did not amplify DNA at all from the 8 genotypes used in the primer screening experiments. Among those primers that amplified DNA fragments from the sorghum templates only one primer (OPA-08) produced a single monomorphic band whereas, the rest produced multiple banding profiles (Figure 1). Most of the RAPD bands obtained were monomorphic. The number of polymorphic bands produced by the selected 40 primers among the 48 genotypes ranged from 1 (as in OPK-15 and UBC-72) to 6 (OPG-05), with the average being 2.7 polymorphisms per primer. In total, 99 polymorphic bands were scored and used in the genetic distance studies.

In this study, the mean frequency of amplification of a polymorphic band was 0.57 ± 0.05. Taking the bands individually, none of them could be used to tag any commercial cultivar. However primer OPAR-14 produced a band (~420bp) that was only unique to genotype 42K.

The RAPD bands were used to calculate genetic distances between genotypes and the average genetic distance for the 1128 inter-pair comparisons was 0.494 ± 0.113, with a range of 0.053 to 0.761. The entire 48 × 48 triangular matrix of genetic distances is too lengthy to be shown here, however part of the matrix is shown in Table 2.

Relationships revealed by MDS analysis

The MDS plot of the genetic distances derived from the 99 polymorphic RAPD bands is shown in Figure 2. This plot was a good fit to the distance matrix since the stress level (the goodness of fit parameter for MDS) was 0.07. A stress level of 0.05 is described as an excellent fit, 0.10 a good fit, 0.2 a fair fit and 0.4 a poor fit (Kruskal, 1964). The sorghum genotypes fall into 2 clusters. The main cluster is made up of cultivated sorghums and the other cluster is made up of the wild sorghums (W9, W10 and W15). Within the major cluster of cultivated sorghums the

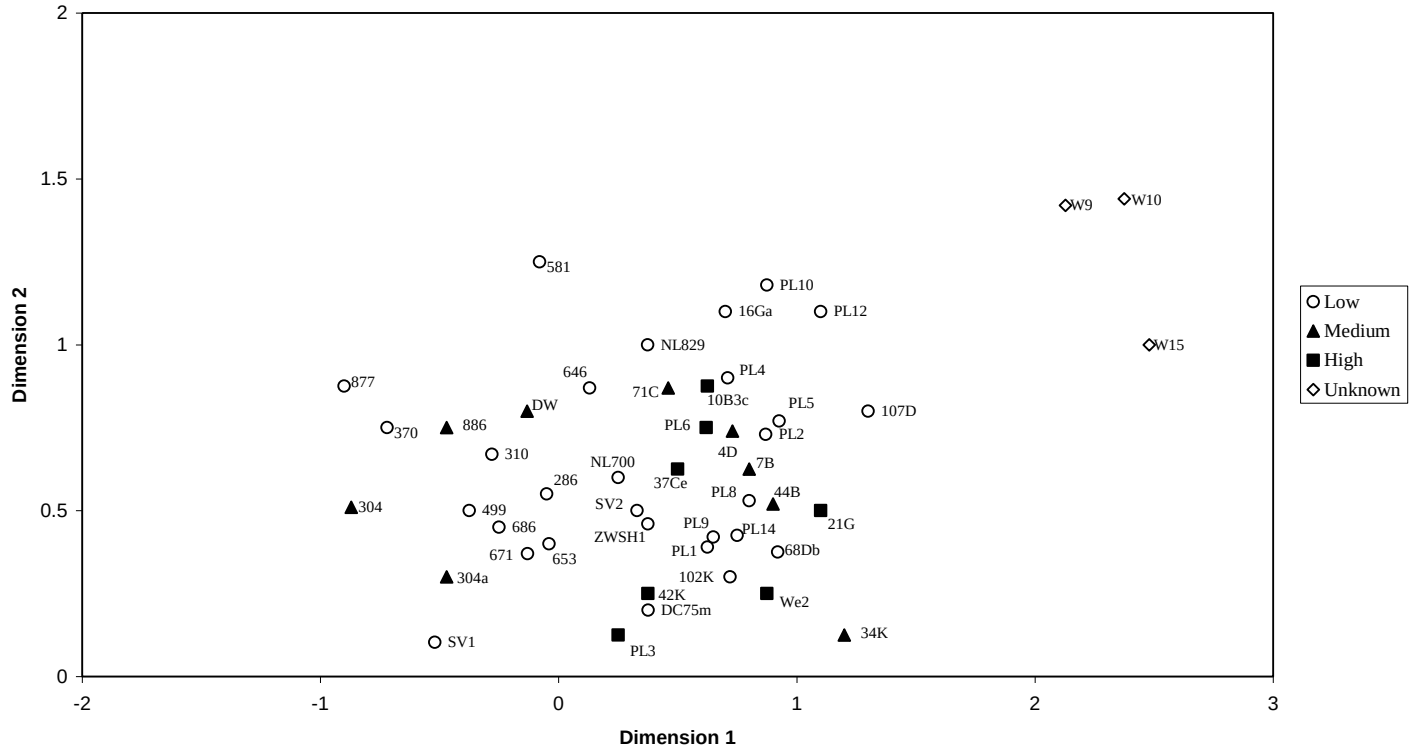

Figure 2. MDS plot of genetic distances of 48 Zimbabwean sorghum cultivars calculated from 99 RAPD bands and classified for their seed proanthocyanidin content as high, medium, low or unknown.

Table 2. Part of the matrix of genetic distances between some pairs of sorghum cultivars used in the study.

10B3c	0.000														
304	0557	0.000													
304a	0.535	0.164	0.000												
34K	0.448	0.565	0.549	0.000											
PL14	0.358	0.521	0.457	0.412	0.000										
4D	0.423	0.514	0.493	0.472	0.188	0.000									
646	0.441	0.616	0.600	0.575	0.382	0.423	0.000								
877	0.608	0.484	0.422	0.611	0.587	0.615	0.563	0.000							
DW	0.493	0.522	0.485	0.493	0.573	0.603	0.569	0.460	0.000						
42K	0.414	0.600	0.560	0.500	0.394	0.453	0.406	0.507	0.493	0.000					
7B	0.338	0.560	0.539	0.414	0.200	0.324	0.408	0.620	0.553	0.397	0.000				
PL9	0.343	0.507	0.443	0.420	0.051	0.200	0.391	0.592	0.541	0.380	0.212	0.000			
PL15	0.421	0.425	0.384	0.405	0.390	0.444	0.442	0.494	0.352	0.368	0.412	0.355	0.000		
16Ga	0.364	0.589	0.534	0.486	0.400	0.438	0.541	0.632	0.562	0.486	0.446	0.431	0.475	0.000	
PL10	0.344	0.583	0.587	0.500	0.414	0.452	0.514	0.679	0.575	0.500	0.371	0.423	0.524	0.297	0.000
	10B3c	***304***	***304a***	***34K***	***PL14***	***4D***	***646***	***877***	***DW***	***42K***	***7B***	***PL9***	***PL15***	***16Ga***	***PL10***

sweet-stem sorghums (877, 886, 370 and 304) group together. The genotypes did not cluster into their PA groups as defined in Table 1. Thus the RAPD markers generated in this study could not distinguish between the low, medium and high PA sorghum cultivars. This was further confirmed by comparing the mean genetic distances of genotypes in different PA groups. If a particular group of genotypes is genetically well differentiated from the other groups, the mean GD among genotypes within the group should be smaller than that between the other groups (Menkir et al., 1997). In this study the mean GDs among the genotypes with high, medium and low

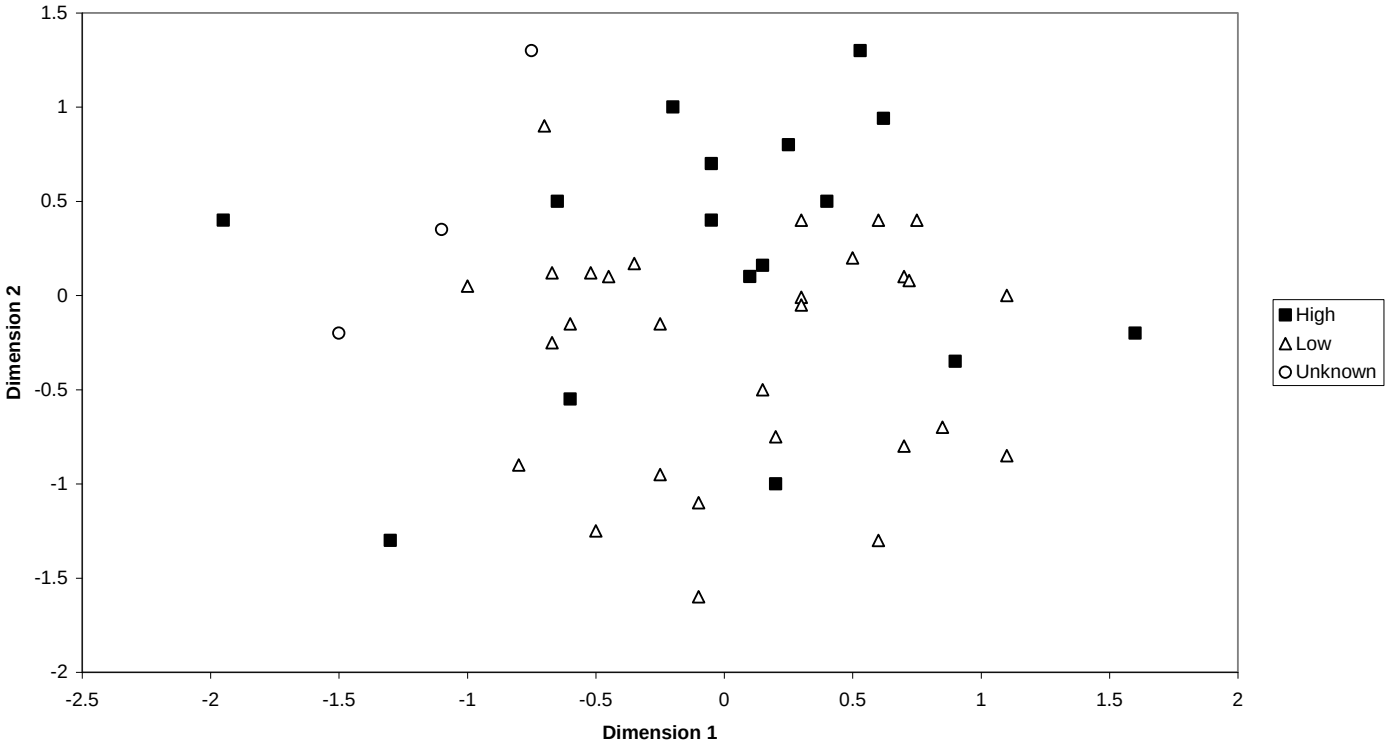

Figure 3. MDS plot of genetic relationships among sorghum genotypes having high and low seed PA levels. Genetic distances were derived from 10 RAPD markers showing the greatest differences between high And low groups in terms of the frequency of amplification within each group.

PA levels which where 0.4802 (± 0.0788); 0.4876 (±0.0533) and 0.4680 (±0.1166), respectively are not significantly different hence we cannot distinguish these groups using the 99 RAPD bands. After maximizing band frequency variances between groups no single RAPD band was found that had an absolute ability to distinguish the groups. Band UBC-180$_{(2000)}$ had the highest separation ability having a frequency variance of 0.220. The majority of the bands had much lower values ranging from 0.0 to 0.128. Fifteen bands with the highest frequency variances between the groups were selected and used to compute genetic distances. These genetic distances still failed to separate the three groups according to their differences in seed PA content (MDS plot not shown). Even after classifying the genotypes as either high or low in PAs and band frequencies between the groups calculated, there still wasn't any band capable of separating the two groups. However, the MDS plot (Figure 3) produced from distances derived from the best 15 bands separated the two groups in a much better way compared to when they were classified into three groups.

DISCUSSION

The genotypes used in this study were collected from different parts of Zimbabwe and the fact that about 35% of these sorghums were high in PAs may be an indication that this random sample was almost representative of the national sorghum germplasm. Obilana (1991), after evaluating a considerable number of Zimbabwean sorghum landraces found that about 33% of the sorghums are high in PAs by looking at seed colour.

The RAPD technique used in this study seems to have been well optimized for sorghum since the average number of polymorphisms detected (2.7 per primer) is within the range of what other groups (Zhan et al., 2012; Agama and Tuinstra, 2003) working on sorghum usually get. The mean genetic distance of about 0.5 between all these genotypes demonstrates adequate coverage of the genome and that there is a substantial level of variation within Zimbabwean sorghums. Furthermore it can be said that the genotypes were discriminated efficiently since the RAPD markers used showed genetic independence, with frequency of amplification for any polymorphic marker being 0.567 (this value must be >0.5 if the RAPD markers are to distinguish genotypes efficiently (Noormohammadi et al., 2012).

Other sorghum studies (Menkir et al., 1997) reveal that there is a considerable variation in the world collection of sorghum and that genotypes from Southern Africa are generally less diverse than those from East and Central Africa. This observation is further substantiated by the absence of clear genetic clusters within the cultivated Zimbabwean sorghum landraces used in this study.

This lack of major genetic subdivisions in this sorghum collection may be an indication that these landraces have

not been significantly isolated in space and thus the introgression of genetic material between them has been occurring over time. Moreover, in the communal farming sector different sorghum landraces are grown in close proximity. The high levels of polymorphisms between some individual genotypes identified in this and other studies may be due to artificial selection for different traits and creation of new genetic combinations by breeders.

This study did not reveal any significant genetic relationships between sorghum cultivars based on their seed PA levels. Since no marker for PAs was found in this population, which was seemingly adequately covered with the RAPD markers used, one can conclude that the trait is controlled by a relatively small portion of the genome otherwise polymorphisms for it could have been identified. Since the MDS plot (Figure 2) does not reveal any PA level group clusters it is concluded that the presence or absence of this trait did not in any way influence the evolution or selection of sorghums over the years, thus sorghums with high PA levels did evolve together with low PA cultivars and have many other traits in common. Furthermore, the observation that MDS plots derived from RAPD markers with maximized variances between the high and low PA groups (Figure 3) revealed a better separation of the genotypes, maybe an indication that the presence or absence of PAs in sorghum seeds is not a polygenic trait. This is in agreement with the results from classical genetics experiments carried out by Woodruff et al., (1982). The RAPD markers for PAs can be obtained if another approach such as use of the near isogenic-lines (NILs) segregating the trait is adopted. The RAPDs protocol established in this work, together with the genetic distance information and PA analysis data
can be used as a quick and cost effective method to prescreen NIL production protocols.

It is possible that the different cultivars are mutants at different structural and regulatory loci controlling flavornoid biosynthesis (Wu et al., 2012). Studying these loci using high throughput genotyping methods such as TILLING (Targeting Induced Local Lesions in Genomes) can aid the development of useful genetic markers for PAs in sorghum (Blomstedt et al., 2011).

REFERENCES

Abbott LA, Bisby FA, Rogers DJ (1985). Taxonomic Analysis in Biology: Computers, Models, and Data Bases. Columbia University Press, New York.

Abdel-Fatah BE, Ali EA, Tag AA, Hessein EM (2013). Genetic diversity among sorghum (*Sorghum bicolor* L. Moench) Landraces in Agro-morphological Traits and Molecular Markers. Asian J. Crop Sci. 5(2): 106-124.

Agama HA, Tuinstra MR (2003). Phylogenetic diversity and relationships among sorghum accessions using SSRs and RAPDs. Afr. J. Biotechnol. 2(10): 334-340.

Blomstedt CK, Gleadow RM, O'Donnell N, Naur P, Jensen K, Laursen T, Olsen CE, Stuart P, Hamill JD, Møller BL (2012). A combined biochemical screen and TILLING approach identifies mutations in *Sorghum bicolor* L. Moench resulting in acyanogenic forage production. Plant Biotechnol. J.10(1): 54-66.

Bretting PK, Widrlechner MP (1995). Genetic markers and horticultural germplasm management. HortScience. 30(7):1349-1356.

Chakauya E, Tongoona P, Matibiri EA, Grum M (2006). Genetic diversity assessment of sorghum landraces in Zimbabwe using microsatellites and indigenous Local Names. Int. J. Bot. 2(1):29-35.

Dudley JW (1994). Comparison of genetic distance estimators using molecular marker data. In: *Analysis of Molecular Marker Data,* Am. Soc. Hortic. Sci., Corvallis, Oregon USA.

Everitt BS (1993). Cluster Analysis 3rd ed. Edward Arnold, London.

FAO (2001). FAO production year-book, 2001, Rome Italy.

Gower JC (1985). Measures of similarity, dissimilarity and distance; In Encyclopedia of Statistical Sciences 5, eds. Kotz. S and Johnson, pp.397 - 405 N. L. Wiley, New York.

Jhingan AK (1992). A novel technology for DNA isolation. Methods Mol. Cell. Biol. 3(15): 15-22.

Kruskal JB (1964). Multidimensional Scaling by optimizing goodness of fit to a non metric hypothesis. Psychometrika 29:1-27.

Menkir A, Goldsbrough P, Ejeta G (1997). RAPD based assessment of genetic diversity in cultivated races of sorghum. Crop Sci. 37: 564-569.

Mohammadi SA, Prasanna BM (2003). Analysis of Genetic Diversity in Crop Plants: Salient Statistical Tools and Considerations. Crop Sci. 43:1235-1248.

Nagaraj N, Sachin M, Surajit H, Ban MCS, Chandrakanth, MG (2013). Baseline Scenario of Postrainy Season Sorghum Economy in Marathwada Region of Maharashtra. Working Paper Series No. 38. Patancheru 502 324, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics. 28 pp.

Nei M, Li W (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Science*s USA 76: 5269-5273.

Ng'uni D, Geleta M, Bryngelsson T (2011). Genetic diversity in sorghum (*Sorghum bicolor* (L.) Moench) accessions of Zambia as revealed by simple sequence repeats (SSR). *Hereditas* 148:52-62.

Nienhuis J, Tivang J, Skroch P (1994). Analysis of genetic relationships among genotypes based on molecular marker data. In: *Analysis of Molecular Marker Data,* American Society for Horticultural Science, Corvallis, Oregon USA.

Nienhuis J, Tivang J, Skroch P, dos Santos JBN (1995). Genetic relationships among cultivars and landraces of lima bean (*Phaseolus lunatus* L.) as measured by RAPD markers. J. Am. Soc. Hortic Sci. 120(2): 300-306.

Noormohammadi Z, Fasihee A, Homaee-Rashidpoor S, Sheidai M, Baraki SG, Mazooji A, Tabatabaee-Ardakani AZ (2012). Genetic variation among Iranian pomegranates (*Punica granatum* L.) using RAPD, ISSR and SSR markers. Aust. J. Crop Sci. 6(2): 268-275.

Obilana AT (1991). Description and evaluation of sorghum germplasm from Zimbabwe. In: *Proceedings of the 21st Anniversary: Crop production Congress,* Harare, Zimbabwe.

SAS Institute 1990 *SAS/STAT User's Guide,* 6th Edition, SAS Institute, Cary, NC, USA..

Schiffman SS, Reynolds ML, Young FW (1981). *Introduction to Multidimensional Scaling*. Academic Press, New York.

Skroch P, Nienhuis J (1995). Qualitative and quantitative characterrization of RAPD variation among snap bean (*Phaseolus vulgaris*) genotypes. Theor. Appl. Genet. 91:1078-1085.

Waniska RD (2000). Structure, phenolic compounds, and antifungal proteins of sorghum caryopses. Pages 72-106 *in* Technical and institutional options for sorghum grain mold management: proceedings of an international consultation, 18-19 May 2000, ICRISAT, Patancheru, India (Chandrashekar A, Bandyopadhyay R, Hall AJ, eds.). Patancheru 502 324, Andhra Pradesh, India: Int. Crops Res. Inst. Semi-Arid Tropics.

Wilkinson L (1992). SYSTAT 5.2: The system for statistics. SYSTAT, Evaston, Illinois, USA

Winkel-Shirley B (2001). Flavonoid Biosynthesis: A colourful model for genetics, biochemistry, cell biology and plant biotechnology. Plant Physiol. 1126: 485-493.

Woodruff BJ, Cantrell RP, Axtell JD, Butler LG (1982). Inheritance of tannin quantity in sorghum. J. Hered. 73:214-218.

Wu Y, Li X, Xiang W, Zhu C, Lin Z, Wu Y, Li J, Pandravadaa S, Ridder DD, Bai G, Wang ML, Trick HN, Scott R. Bean SR, Tuinstra MR,

Tesfaye T. Tesso TT, and Yu J (2012). Presence of tannins in sorghum grains is conditioned by different natural alleles of Tannin1. PNAS 109(26):10281-10286

Zhan Q, Zhou L, Bi N, Wu H, Li J, Lu J, Lin P (2012). A Comprehensive Analysis of Genetic Diversity and Relationships of 13 Sweet Sorghum Varieties as Energy Sources. J. Sust. Bioenergy Syst. 2(4):86-91.

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Short Communication

Genotypic frequency of *calpastatin* gene in lori sheep by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method

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Calpastatin is a natural occurring inhibitor of calpastatin (CAST) and consequently the balance of calpain-calpastatin activity in muscles is believed to dictate the rate of tenderization in post-mortem meat. Genomic DNA was extracted from 100 sheep blood sample. Polymerase chain reaction was performed to amplify a 622 bp fragment of this gene. Restriction reaction of polymerase chain reaction (PCR) products was done using MspI enzyme. The MspI digestion of the PCR products produced digestion fragments of 336 and 286 bp. The results show that in the population, genotypes AA, AB and BB, respectively, had frequencies 32.2, 63.2 and 4.6, and that this locus was not at Hardy - Weinberg equilibrium in the lori sheep strain (P<0.05).

Key words: *Calpastatin* gene, polymorphism, lori sheep, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

INTRODUCTION

Increase in sheep production will help increase mutton production and study on *calpastatin* gene combined with other molecular techniques such as marker assisted selection (MAS) can play very important part to better sheep production in Iran. The effect of calpains gene polymorphism on the analyses meat quality traits are discussed in detail in another paper (Goll et al., 1998; Chung et al., 2002; Forsberg et al 1989). The protein encoded by this gene is an endogenous calpain (calcium-dependent cysteine protease) inhibitor. It consists of an N-terminal domain L and four repetitive calpain-inhibition domains (domains 1-4), and it is involved in the proteolysis of amyloid precursor protein. Of the five domains, the N-terminal leader (L) domain does not appear to have any calpains inhibitory activity, but maybe involved in targeting or intracellular localization (Takano et al., 1999), while the other domains (I-IV) are highly homologous and are each independently capable of

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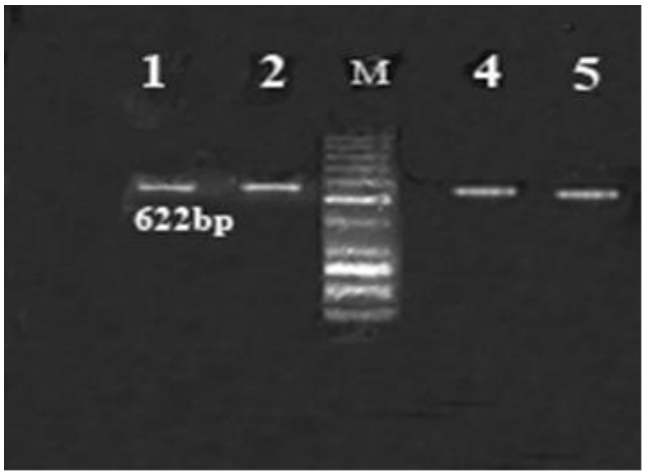

Figure 1. PCR product analyzed by electrophoresis (622 bp).

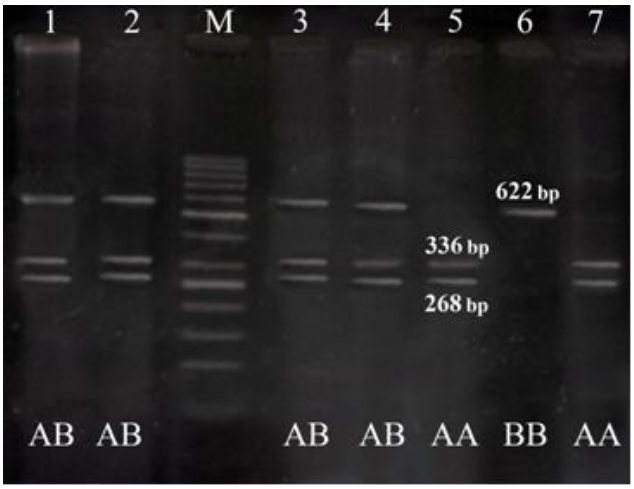

Figure 2. Genotype AA, AB and BB digestion with MspI.

inhibiting calpains (Cong et al., 1998). This Indicates that the inhibitory domains of calpastatin contain three highly conserved regions, A, B and C, of which A, played a regulatory role by altering phosphorylation patterns on the protein (Takano et al., 1999). Calpastatin (*CAST*) gene is located on the fifth chromosome of sheep and plays important roles in the formation of muscles, degradation and meat tenderness after slaughter. Increased rate of skeletal muscle growth can result from a decreesed rate of muscle protein degradation, and this is associated with a decrease in activity of the calpain system, due principally to a large increase in calpastatin activity (Goll et al., 1998).

Associations have been reported between variation in CAST, and carcass and meat quality traits in cattle (Casas et al., 2006; Schenkel et al., 2006), but in sheep, a genetic variation in the CAST gene has been investigated too (Palmer et al., 2000; Zhou et al., 2007). In our research, we have studied the position of the *calpastatin* gene in the lori sheep breeds in Iran.

MATERIALS AND METHODS

In this study, random blood samples were collected from 100 lori sheep from different regions in Lorestan province of Iran. Approximately, 5 ml blood sample was gathered from vena in ethylene diaminetetraacetic acid (EDTA) tube and was transferred to -20°C freezer. Genomic DNA was extracted from whole blood. Exon and intron region from a portion of the first repetitive domain of the ovine calpastatin gene were amplified to a product of 622 bp using primers based on the sequence of the bovine (Killefer and Koohmaraie, 1994; Gen bank accession no L14450) and ovine calpastatin genes. In this research, DNA primers described by Palmer (1998) were used for PCR amplification; primers were obtained from Cinnagen Company in a lyophilized form (non-sensitive to temperature).

F:5'-TGGGGCCCAATGACGCCATCGATG-3'
R:5'-GGTGGAGCAGCACTTCTGATCACC-3'

The polymerase chain reaction (PCR) was performed using a buffer PCR 1X, 200 µM dNTPs, 1.5 µM $MgCL_2$, 10 pmol each primer, 1.25 U taq DNA polymerase, 50 ng ovine gnomic DNA and H_2O up to a total volume of 25 µl. 33 cycle of preliminary denaturation at 95°C (5 min), denaturation at 94°C (1 min), annealing at 60°C (1 min), extension at 72°C (2 min) and final extension at 72°C (8 min). The PCR products were separated by 1.2% (w/v) agarose gel electrophoresis. The amplified fragment of calpastatin was digested with *Msp*I. 15 µl of PCR production with 2 µl buffer, U (0.5) of *Msp*I and 11.5 µl H_2O up to a total volume of 29 µl, following the manufacturers instruction for 12-16 h at 37°C. The digestion products were electrophoresed on 2% agarose gel in 1X TBE and visualized by ethidium bromide staining for 1 h at 85 V. Estimates genotype and alleles frequencies and Hardy-Weinberg equilibrium was analysis with Pop Gene 32 package (Yeh et al., 1999).The relative frequency of particular allele in a population is called the allele frequency (Nei and Kumar, 2000).

Description:
$\chi 2$ = Hardy-Weinberg equilibrium test
O = observed number of genotype A11
E = expected number of genotype A11

RESULTS AND DISCUSSION

The amplified calpastatin resulted in a DNA fragment with 622 bp including the sequences of exon and intron regions from a portion with PCR technique (Figure 1). Due to the digestion of 622 bp PCR product for *CAST* gene with restriction endonucleases MspI, three different genotypes were observed (AA, BB and AB). The first genotype (AA) showed the two band pattern (bands of ~336 and 286 bp). In the second genotype (AB), due to a mutation in one of the alleles, bands 622, 336 and 286 bp were observed. In the third genotype (BB), one band pattern (~ 622 was observed (Figure 2).

This result shows that the polymorphism was detected in CAST I segment, as previously reported in a variety of other sheep in the world such as the dorset sheep (Palmer

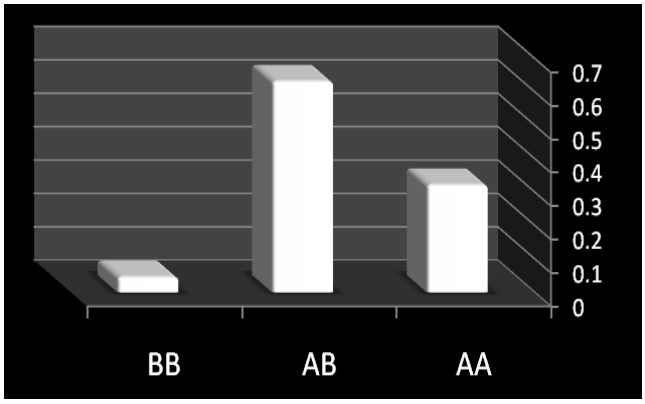

Figure 3. Genotype frequencies of the calpastatin in lori sheep.

Table 1. Chi-square test of the *calpastatin* gene in lori sheep.

Genotype	Observed frequency	Expected frequency	p
AA	0.322	0.407	
AB	0.362	0.462	0.001281 **
BB	0.044	0.131	

et al., 1998), Kurdi sheep in Iran (Nassiry et al., 2006), Merino, Corriedale, Romney, Poll Dorset, and crossbred NZ sheep in New Zealand (Zhou et al., 2007) and Sutikno (2011), and Ghezel sheep (Elyasi et al 2009). After assessment of the samples, the frequencies of A and B alleles were calculated as 0.638 and 0.362, respectively. Also the frequencies of AA, AB and BB genotypes were calculated as 0.332, 0.632 and 0.046, respectively (Figure 3). In their present researches, the fragments size, the number of alleles and genotypes observed were similar to those of Palmer et al. (1998). They reported the three fragments of 286 and 336 bp length, and hence two alleles with three different genotypes. According to the obtained data analysis in their present researches, the results were significant in both tests used and sheep populations were not in Hardy Weinberg equilibrium (Table 1).

Hardy-Weinberg equilibrium can be affected by inbreeding, assortative mating, natural selection and population subdivision (Nei and Kumar, 2000). Lack of Hardy Weinberg equilibrium for *calpastatin* gene in other populations have been reported by researchers (Elyasi et al., 2009; Mohammadi et al., 2008) and (Gabor et al., 2009). The results indicate that it could be useful to consider genetic diversity at calpastatin locus in lori sheep.

Conflict of Interests

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

REFERENCES

Chung HY, Kim CD, Cho CY, Yeon SH, Jin HJ, Jeon KJ, Kim HC, Lee HJ, Hines HC, Davis ME (2002). Effects of calpastatin gene polymorphisms on growth and carcass traits of Korean Native cattle. Proceedings of the 7th World Congress on Genetics Applied to Livestock Production.

Elyasi J, Shoja M, Nassiry MR, Pirahary O, Javanmard A (2009). Allelic and genotypic frequency of Calpastatin gene in Ghezel and Arkhamerino sheeps and their crossbreds. J. New Agric. Sci. 4, No. 13.

Forsberg NE, Ilian MA, Ali-Bar A, Cheeke PR, Wehr NB (1989). Effects of cimaterol on rabbit growth and myofibrillar protein degradation and on calcium dependent proteinase and calpastatin activities in skeletal muscle. J. Anim. Sci. 67: 3313-3321.

Goll DE, Thompson VF, Taylor RG, Ouali A (1998). The calpain system and skeletal muscle growth. Can. J. Anim. Sci. 78: 503-512.

Killefer J, Koohmaraie M (1994). Bovine skeletal muscle calpastatin: cloning, sequence analysis, and steady-state mRNA expression. J. Anim. Sci. 72: 606-620.

Nei M, Kumar S (2000). Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Nassiry MR, Mojtaba T, Ali S, Mahdi F, Saheb F (2006). Calpastatin polymorphism and its association with daily gain in Kurdi sheep. J. Iran. Biotechnol. 4: 188-192.

Palmer BR, Roberts N, Hickford JG, Bickerstaffe R (1998). Rapid Communication: PCR-RFLP for MSP I and Noc I in ovine calpastation Gene. J. Anim. Sci. 76:1499-14500.

Palmer B R, Su HY, Roberts N, Hickford J G, Bickerstaffe R (2000). Single nucleotide polymorphisms in an intron of the ovine calpastatin gene. Anim. Biotechnol. 11, 63–67

Sutikno B, Yaminc M, Sumantri C (2011). Association of Polymorphisms Calpastatin Gene with Body Weight of Local Sheep in Jonggol, Indonesia. Media Peternakan, hlm. 1-6 EISSN 2087-4634.

Takano J, Kawamura T, Murase M, Hitomi K, Maki M (1999). Structure of mouse calpastatin isoforms: implications of species-common and species-specific alternative splicing. Biochem. Biophys. Res. Commun. 260: 339-345.

Yeh F, Yang C, Boyle T (1999). POPGENE version 1.31 Microsoft window-based freeware for Population Genetic Analysis, University of Alberta. Edmonton, AB. Canada.

Zhou H, Hickford JGH, Gong H (2007). Short communication: Polymorphism of the ovine calpastatin gene. J. Mol. Cell. Prob. 21: 242–244.

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Full Length Research Paper

Effect of priming on germinability and salt tolerance in seeds and seedlings of *Physalis peruviana* L.

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In some species, pre-germination treatments such as priming can increase germinability and the speed of the process, besides conferring tolerance to abiotic stress. The central effect of priming is the slow and controlled absorption of water in seed tissues, allowing the membranes to reorganize and synthesize protective substances against stress. This study was performed to assess the effects of priming on the invigoration of seeds and seedlings of *Physalis peruviana* subjected to salt stress. Seeds of *P. peruviana* were primed in polyethylene glycol 6000 to -0.8 MPa and were germinated in solutions with different salt concentrations (0, 4, 8 and 12 dS m^{-1}). In addition to the rate of radical emergence, post-seminal development was also evaluated until the emergence of the cotyledons. Germinability decreased with increasing salt solution concentrations in both types of seeds. Priming appeared to alleviate the effects of salt stress in the early stages of development of *P. peruviana*. Total dry mass of seedlings increased under saline conditions, suggesting possible physiological adjustments induced by priming.

Key words: Germination, priming, salt stress, Solanaceae.

INTRODUCTION

For many plant species, seeds are the main means of dispersal. Germination and seedling establishment are the most sensitive stages of development with regards to environmental conditions, and affect the expansion of a species in a new environment (Natale et al., 2010). However, abiotic factors such as water availability and salinity of soil water drastically affect this process. Plants need minerals to grow and develop; however, salt in excess can be extremely dangerous to plants (Xiong and Zhu, 2002). The successful development of some species subjected to salt stress depends on their ability species to tolerate such conditions. Seeds with better germinality and salt tolerance can survive more effectively. Priming has been recommended as a pre-germination treatment for the production of cultivars in order to increase the rate of germination and seedling

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Abbreviations: NP, Non-primed; **P,** primed; **PEG,** polyethylene glycol.

establishment under adverse conditions (Heydecker and Coolbear, 1977; Bradford, 1986; Nakaune et al., 2012). This technique involves the absorption of water by the seeds followed by drying and rehydration when the early stages of germination begin, at radicle protrusion (Basu, 1994; McDonald, 2000). Besides increasing germination rate, the benefits of this technique include an increase in uniformity and germinative process. According to De Castro et al. (2000), the effects of priming are prolonged growth and vigour of the seedling. This technique has been successfully used in crops such as wheat (Iqbal and Ashraf, 2007), chickpeas (Kaur et al., 2002), sunflowers (Kaya et al., 2006) and cotton (Casenave and Toselli, 2007).

Physalis peruviana L. (family Solanaceae) is a native plant of the Andes and it is produced on a large scale in Colombia and South Africa. The species is known as a medicinal herb in Peru (Fischer et al., 2007; Mazorra et al., 2006). Its fruit is considered a good source of natural anti-oxidants and other components, e.g. vitamins A, B, C, E and K1, phytosterols and essential minerals (Puente et al., 2011).

In Brazil, the fruit of *P. peruviana* L. is considered exotic and has a high market value (Lima et al., 2009). The fruit ripens and acquires an orange color, and its persistent calyx becomes papery and light brown. This adds aesthetic value to the fruit which is sold primarily for decoration of fine pastries, pies and jams or as a fresh fruit. In Brazil other species of the genus, such as *Physalis angulata* L., have been widely studied in the Northeast region, primarily for their medicinal potential (Souza et al., 2013).

There is potential for the cultivation of this species in arid regions where excessive salt in the soil has been a limiting factor in agriculture. Studies on the effect of salinity on crop growth are gaining importance, and our understanding of how the genus *Physalis* responds to such conditions can add to this. Thus, this study assesses the effect of seed priming on salinity tolerance during germination and early development of *P. peruviana*.

MATERIALS AND METHODS

The seeds of *P. peruviana* used in the study were collected in June 2011, at the Horto Florestal Station at the Universidade Estadual de Feira de Santana, Bahia, Brazil. They were removed from the fruit under running water until complete separation of the pulp. The seeds were dried in a desiccator containing super-saturated calcium chloride solution at 20°C. Water content was determined on a fresh weight basis by placing samples of 200 seeds for 17 h in an oven set at 103°C (ISTA, 2007). Remaining seeds were placed in 5 ml tubes and stored at 4°C until further use.

Samples of 500 seeds of *P. peruviana* were primed by immersion in polyethylene glycol solution ("PEG 6000"), with an osmotic potential of -0.8 MPa, (Villela et al., 1991). This priming potential was established according to the method developed for *P. angulata* by Souza et al. (2011). The immersion was performed in 20 ml test-tubes coupled with an artificial aeration system for 10 days at 25°C in a germination chamber, adjusted for a 12 h photoperiod. The osmotic solution was replaced every two days until day 10 when, after the pre-germination period, the seeds were taken from the solution and dried in an incubator saturated with calcium chloride solution at 20°C for 4 h until the initial weight was reached.

For the germination experiments, 400 primed (P) and 400 non-primed (NP) seeds were placed in Petri dishes made of glass containing two sheets of germitest paper (sterilized in a drying oven at 105°C for 4 h), moistened with solutions of different NaCl concentrations (0, 4, 8 and 12 dS m^{-1}). The concentration of each solution was measured by electrical conductivity. The seeds were kept in the germination chamber at 25°C, adjusted to a 12 h photoperiod for 30 days. Daily observations were performed, with seeds those that issued a radicle of at least 1 mm. recorded as "germinated." Each treatment consisted of four batches of 25 seeds.

The parameters assessed for germination were germinability (%), relative frequency (%), average speed of germination (day^{-1}) germination speed index of seeds (GSI) (seed.day^{-1}) and coefficient of uniformity of germination (CUG). Daily the germinated seeds were transferred to another Petri dish moistened with solutions of different NaCl concentrations (0, 4, 8 and 12 dS m^{-1}) for 10 days for analysis of post-seminal development. Seedlings considered normal according to internationally standardized rules (ISTA, 2007), had the length of the radicle and shoots (from the insertion of the cotyledons) measured with the aid of a digital caliper and placed in a forced-air circulation oven at 40°C for 10 days to dry. After this period, the samples were stored in a desiccator with silica and subsequently weighed on a precision balance. The samples ranged according to the germinability and normal seedlings per treatment. The mass per replicate was determined in mg/seedling. To analyze the post-seminal development, normal seedlings (%), total dry mass of normal seedlings (mg/seedling) and ratio of the length radicle/shoots were assessed. Data analysis of germination and post-seminal development were performed using the SISVAR computer program (Ferreira, 2011).

RESULTS

Germinability (%) of primed and non-primed seeds of *P. peruviana* showed a negative quadratic trend, with a decrease in the percentage as the concentration of the salt solutions increased (Figure 1). In the solution with electrical conductivity (EC) of 8 dS m^{-1} seeds showed an 89% rate of germination when compared to non-primed seeds with a rate of 58%. At the highest salt concentration used in this assay (12 dS m^{-1}) 41% of the primed seeds of *P. peruviana* germinated *versus* 31% of the non-primed seeds.

The germination speed index (GSI) increased signifi-cantly in primed seeds (4.12) grown in a control solution (0 dS m^{-1}) compared to non-primed (3.4 seed.day^{-1}) and to salt concentration with EC of 8 dS m^{-1} (2.4 and 1.3 seed.day^{-1}, respectively) (Table 1).

The relative frequency polygon was polimodal in solutions with electrical conductivity of 12 dS m^{-1} and unimodal in other concentrations (Figure 2). In unimodal polygons, the peaks were higher in primed seeds versus non-primed, reflecting a greater uniformity of germination, supported by the uniformity coefficients presented in control treatment (1.520 and 0.664, respectively).

During post-seminal post evaluation normal seedling development of *P. peruviana* took place under low salinity conditions (Figure 3A). The highest number of normal seedlings came from primed seeds, regardless of electrical conductivity (66.7 and 61.5% at EC of 0 dS m^{-1}), and this difference was significant for those kept in saline

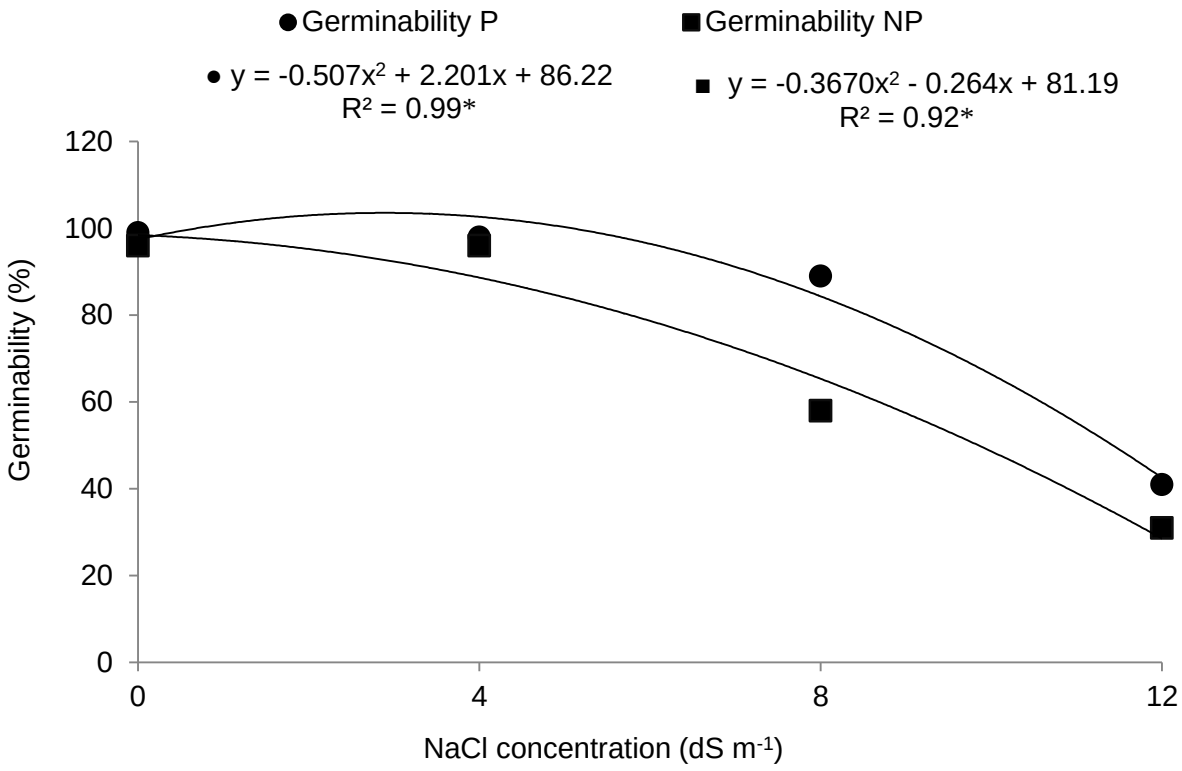

Figure 1. Germinability of seeds of *P. peruviana* L. originated from primed (P) and non-primed (NP) seeds. *Significant at 5% probability.

Table 1. Average speed (day^{-1}), germination speed index (GSI, seeds.day^{-1}) and coefficient of uniformity of germination (CUG) of primed (P) and non-primed (NP) seeds of *Physalis peruviana* L. under different salt concentrations.

Seed	NaCl concentration (dS m^{-1})			
	0	4	8	12
Average speed (day^{-1})				
P	0.163^{a}	0.120^{b}	0.100^{b}	0.081^{c}
NP	0.137^{a}	0.119^{a}	0.084^{b}	0.051^{c}
GSI (seeds. day^{-1})				
P	4.116^{a}*	3.090^{b}	2.365^{b}*	0.846^{c}
NP	3.429^{a}	2.963^{a}	1.300^{b}	0.586^{c}
CUG				
P	1.520^{a}	0.388^{a}	0.199^{a}	0.306^{a}
NP	0.664^{a}	0.540^{a}	0.138^{a}	0.086^{a}

Means followed by the same letter in the line are not significantly different according Tukey test, at 5% probability. *Significant by Student's t-test at 5% probability.

(70.5 and 48.1 at EC of 4 dS m^{-1}).

Data from the root:shoot ratio demonstrated that seedlings from primed seeds showed a radicle length 3.15-fold greater than the shoots kept in the control solution (0 dS m^{-1}). However, in seedlings from non-primed seeds this value was only 1.58, and this difference is significant (Figure 4). Seedlings subjected to salt stress had shoots of a greater length for both seedlings, whether derived from primed or non-primed seeds (0.55 and 0.57). Under saline conditions, although the total dry mass increased, the allocation of this mass was not influenced by priming.

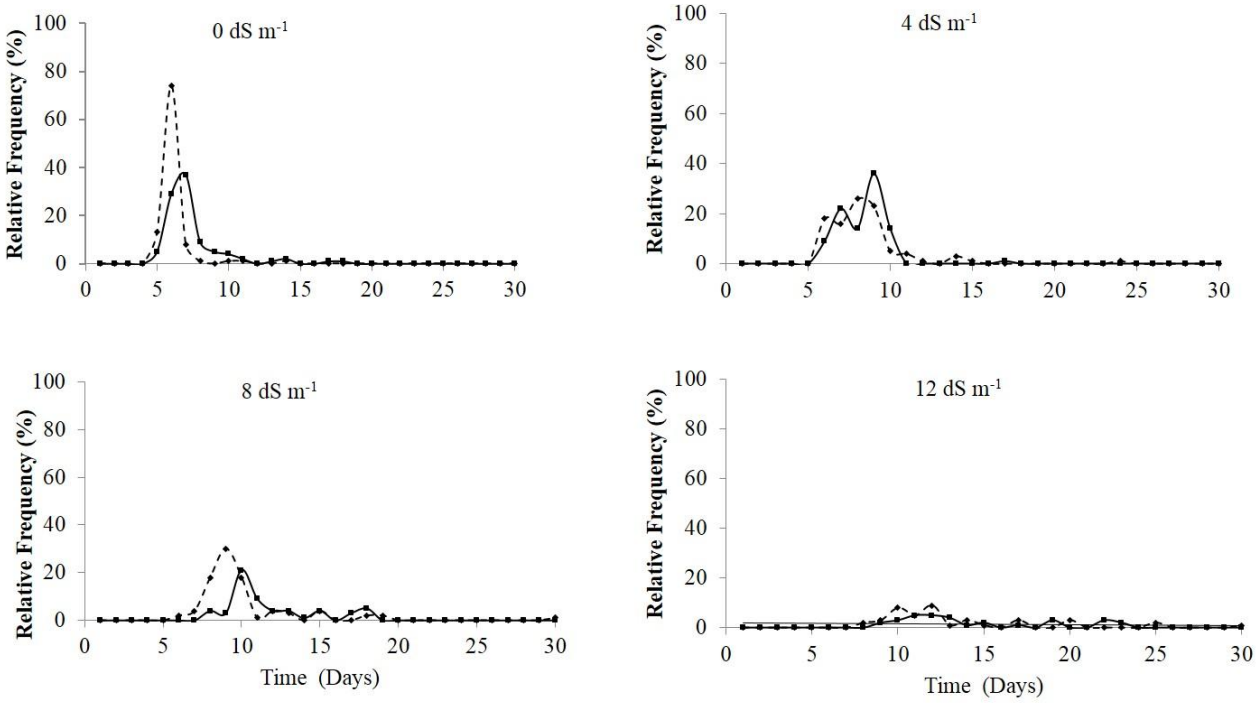

Figure 2. Relative frequency polygons (FR%) of germination of *Physalis peruviana* L. for different salt concentrations. P (- -♦- -) e NP (—■—) .

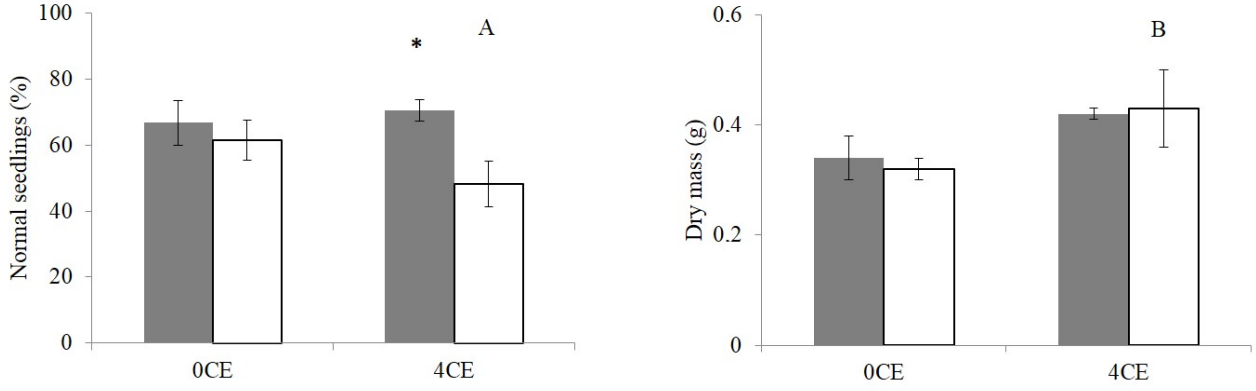

Figure 3. Percentage (A) and dry mass (B) of normal seedlings of *Physalis peruviana* L. from P (■) and NP (□) seeds and post-germination in water and saline (4 EC). *Significant at 5% probability.

DISCUSSION

The 31% difference in germinability between primed and non-primed seeds in saline with EC of 8 dS m^{-1} demonstrates that priming induced higher salinity tolerance. During priming the metabolic energy in primed seeds is greater than in non-primed seeds because increases in adenosine triphosphate (ATP), energy charge (EC) and ATP/ADP (adenosine diphosphate) ratio (Corbineau et al., 2000; Varier et al., 2010).

Investigations using *P. angulata* have also demonstrated that priming provides salt tolerance, even at high concentrations (Souza et al., 2011). However, at the highest salt concentration (12 dS m^{-1}) used in the present assay, seeds of *P. peruviana* demonstrated around half the germination success of those of *P. angulata* (41% compared with 83%, respectively), when kept in saline with the same electrical conductivity.

Despite belonging to the same genus, *P. angulata* and *P. peruviana* responded differently to the priming technique, with a more significant effect on salt tolerance in *P. angulata*. However, to a lesser extent, the benefits

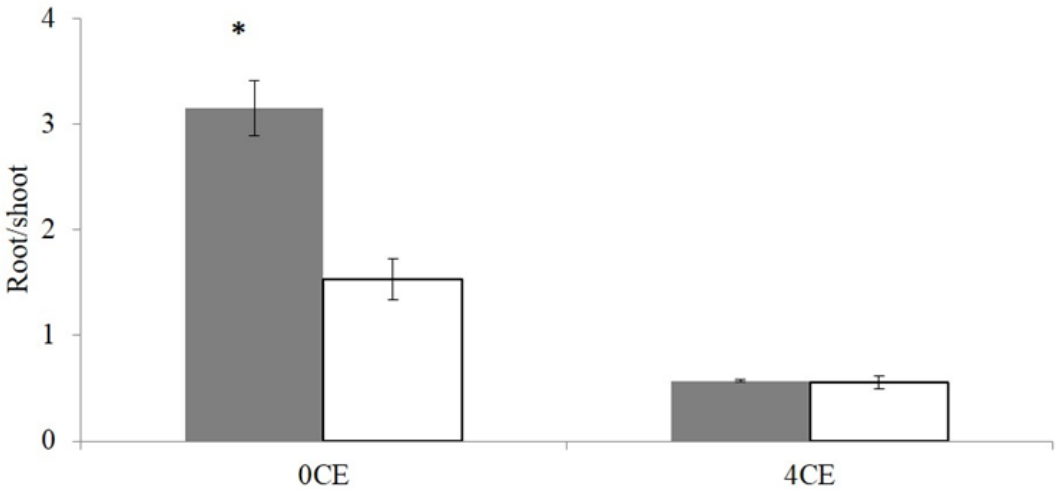

Figure 4. Ratio of length between root and shoot of *Physalis peruviana* L. seedlings from P (■) and NP (□) seeds and posterior germination in water and saline (4 EC). *Significant at 5% probability.

of priming were also observed in *P. peruviana*, which confirms that this technique can be used as pre-germination treatment.

Yildirim et al. (2011) observed that in seeds of *P. peruviana* and *Physalis ixocarpa* the germinability (%) decreased, and the average time increased, as the concentration of saline the seeds were treated with increased. According to the authors, *P. peruviana* is tolerant to salinity during germination but it becomes sensitive during seedling development.

Germinability (%), germination speed index (seeds.day^{-1}) and average speed (day^{-1}) results observed in the present study are corroborated by Bradford (1986) and Nakaune et al. (2012). They claim that, under saline conditions, priming has a protective effect on oxidative damage caused by the accumulation of sodium in the cell cytoplasm.

Data from seedling dry mass did not agree with those presented by Yildirim et al. (2011), which indicated that the salt stress affected the seedlings of *P. peruviana* and *P. ixocarpa* adversely, with a decrease in dry mass as salt concentration increased. However, according to Miranda et al. (2010), plants of *P. peruviana* showed a positive effect when subjected to saline at a concentration of 30 mM (around 4 dS m^{-1}). According to these authors, *P. peruviana* is moderately salt tolerant, since the relative growth rate was stimulated by moderate salt stress (30 mM, around 4 dS m^{-1}). This suggests possible physiological and osmotic adjustments to maintain the water potential in the tissues of the plant (Miranda et al., 2010).

As priming is a technique that promotes greater speed and uniformity of germination, the positive effects were also observed in the increase in the root:shoot ratio. Primed seeds produced a radicle in a shorter time than non-primed seeds. We suggest that cell elongation was also faster, reflected in the fact that the radicles from primed seedlings had a 3.15-fold greater length than the shoots. According to De Castro et al. (2000), the activation of genes that occurs during priming is maintained beyond the seed and seedling stages, lasting into the adult stage. As for the decrease in root length in seedlings kept in saline (4 dSm^{-1}), this could relate to a strategy developed by plants under such conditions to limit the absorption of toxic ions such as Na^+ and Cl^- (Munns, 2002; Alarcón et al., 2006).

Priming was effective in the invigoration of seeds and seedlings of *Physalis peruviana* under different saline conditions; however, this response was less effective when compared to other species of the genus in the same conditions, especially during post-seminal development.

Conflict of Interests

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

REFERENCES

Alarcón JJ, Morales MA, Ferrández T, Sánchez-Blanco MJ (2006). Effects of water and salt stresses on growth, water relations and gas exchange in *Rosmarinus officinalis*. J. Hortic. Sci. Biotechnol. 81: 845-853.

Basu RN (1994). An appraisal of research on wet and dry physiological seed treatments and their applicability with special reference to tropical and subtropical countries. Seed Sci. Technol. 22: 107–126.

Bradford KJ (1986). Manipulation of seed water relations via osmotic priming to improve germination under stress conditions. HortScience. 21: 1105-1112.

Casenave EC, Toselli ME (2007). Hydropriming as a pre-treatment for cotton germination under thermal and water stress conditions. Seed Sci. Technol. 35: 88-98.

Corbineau F, Ozbingol N, Vineland D, Come D (2000). Improvement of tomato seed germination by osmopriming as related to energy metabolism. In Black M, Bradford KJ, Vasquez-Ramos J (Eds). Seed Biology Advances and Applications: Proceedings of the Sixth International Workshop on Seeds, Mérida, Mexico, 1999. New York, NY : CABI. 467-474.

De Castro RD, Van Lammeren AAM, Groot SPC, Bino RJ, Hilhorst

HWM (2000). Cell division and subsequent radicle protrusion in tomato seeds are inhibited by osmotic stress but DNA synthesis and formation of microtubular cytoskeleton are not. Plant Physiol. 122: 327-335.
Fischer G, Ebert G, Lüdders P (2007). Production, seeds and carbohydrate contents of cape gooseberry (*Physalis peruviana* L.) fruits grown at two contrasting Colombian altitudes. J. Appl. Bot. Food Qual. 81: 29-35.
Ferreira DF (2011). Sisvar: a computer statistic alanalysis system. Cienc. Agrotecnol. 35: 1039-1042.
Heydecker W, Coolbear P (1977). Seed treatments for improved performance - survey and attempted prognosis. Seed Sci. Technol. 5: 353-425.
Iqbal M, Ashraf M (2007). Seed treatment with auxins modulates growth and ion partitioning in salt-stressed wheat plants. J. Integr. Plant Biol. 49: 1003-1015.
ISTA (2007) International Rules for Seed Testing Association, Bassersdorf, Switzerland.
Kaya MD, Okçu G, Atak M, Çikili Y, Kolsarici O (2006). Seed treatments to overcome salt and drought stress during germination in sunflower (*Helianthus annuus* L.) Eur. J. Agron. 24: 291-295.
Kaur S, Gupta AK, Kaur N (2002). Effect of osmo and hydropriming of chickpea seeds on seedling growth and carbohydrate metabolism under water deficit stress. J. Plant Growth Regul. 37: 17-22.
Lima CSM, Manica-Berto R, Silva SJP, Betemps DL, Rufato AR (2009). Establishment and operating costs of a cape gooseberry orchard in the south region of Rio Grande do Sul. Rev. Ceres. 56: 555-561.
Mazorra MF, Quintana AP, Miranda D, Fischer G, De Valencia MC (2006). Anatomic Aspects of Formation and Growth of the Cape Gooseberry Fruit *Physalis peruviana* (Solanaceae). Acta Biol.Colomb.11: 69-81.
McDonald MB (2000). Seed priming. In: Black M, Bewley JD (Eds). Seed Technology and Its Biological Basis. Sheffield: Sheffield Academic Press. UK. 287-325.
Miranda D, Fischer G, Ulrichs C (2010). Growth of cape gooseberry (*Physalis peruviana* L.) plants affected by salinity. J. Appl. Bot. Food Qual. 83: 175-181.
Munns R (2002). Comparative physiology of salt and water stress. Plant Cell Environ. 25: 239-250.
Nakaune M, Hanada A, Yin YG, Matsukura C, Yamaguchi S, Ezura H (2012). Molecular and physiological dissection of enhanced seed germination using short-term low-concentration salt seed priming in tomato. Plant Physiol. Biochem. 52: 28-37.
Natale E, Zalba SM, Oggero A, Reinoso H (2010). Establishment of *Tamarix ramosissima* under different conditions of salinity and water availability: Implications for its management as an invasive species. J. Arid Environ. 7: 1399 -1407.
Puente LA, Pinto-Muñoz CA, Castro ES, Cortés M (2011). *Physalis peruviana* Linnaeus, the multiple properties of a highliy functional fruit: a review. Food Res. Int. 44: 1733-1740.
Souza MO, Souza CLM, Pelacani CR (2011). Germination of osmoprimed and non-osmoprimed seeds and initial growth of *Physalis angulata* (Solanaceae) in saline environments. Acta Bot. Bras. 25: 105-112.
Souza MO, Souza CLM, Pelacani CR, Soares M, Mazzei JL, Ribeiro IM, Rodrigues CP, Tomassini TCB (2013). Osmotic priming effects on emergence of *Physalis angulata* and the influence of abiotic stresses on physalin content. S. Afr. J. Bot. 88: 191-197.
Varier A, Vari AK, Dadlani M (2010). The subcellular basis of seed priming. Curr. Sci. 99: 450-456.
Villela FA, Doni Filho LD, Sequeira EL (1991). Table of osmotic potential as a function of polyethileneglycol 6000 concentretion and temperature. Pesqui. Agropecu. Bras. 26: 1957-1968.
Xiong L, Zhu JK (2002). Molecular and genetic aspects of plant responses to osmotic stress. Plant Cell Environ. 25: 131-139.
Yildirim E, Karlidag H, Dursun A (2011). Salt tolerance of *Physalis* during germination and seedling growth. Pak. J. Bot. 43: 2673-2676.

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Full Length Research Paper

Effect of nodal positions, seasonal variations, shoot clump and growth regulators on micropropagation of commercially important bamboo, *Bambusa nutans* Wall. ex. Munro

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An efficient protocol for *in vitro* micropropagation of *Bambusa nutans* Wall. ex. Munro has been described. Nodal explants obtained from 1½-year-old field-grown culms of *B. nutans* produced up to 7.0 multiple shoots per explant on Murashige and Skoog (MS) basal medium supplemented with 6-benzylaminopurine (BAP, 1.0 mg/L). Continuous shoot proliferation up to 11.33 shoots was achieved by sub-culturing shoot clumps (4 shoots/cluster) in BAP (0.5 mg/L) and 0.1 mg/l α-naphthalene acetic acid (NAA) fortified medium every 4 weeks. 85% rooting was recorded on 2.0 mg/L NAA supplemented medium after 30 to 35 days of culture period. Micropropagated plantlets of *B. nutans* showed 70% survivability during the hardening stage. After hardening, rooted plantlets were successfully transferred to the soil and exhibited 80% survivability and normal growth. Plantlets cultivated in field condition achieved 95% survivability. Seed explants were also used for *in vitro* culture establishment of *B. nutans* on different combination of MS medium.

Key words: *Bambusa nutans*, micropropagation, nodal explants, seed explants.

INTRODUCTION

Bambusa nutans Wall. ex. Munro (local name Mokal bah) is naturally occurring in sub-Himalayan tracts from Yamuna eastwards to Arunachal Pradesh between 600 to 1500 m of altitude. It is very common in India and Nepal and widely cultivated in the villages of Bangladesh (Banik, 1987) and also reported as an important commercial species of Thailand (Anantachote, 1987). It is a graceful medium-sized, thick-walled bamboo with 6 to 15 m culms height, 5 to 10 cm in stem girth, and usually with 25 to 45 cm long internodes. Among the sympodial bamboos, *B. nutans* produced highest number of culms per clump. This bamboo is reported to be useful for

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Abbreviations: BAP, 6-Benzylaminopurine; **GA_3,** gibberellic acid; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **Kin,** kinetin; **MS,** Murashige and Skoog medium; **NAA,** α-Naphthalene acetic acid; **2ip,** 2-isopentenyladenine; **PGR,** plant growth regulators.

cellulose (paper) manufacture and for various purposes, including pulping, timber, handicrafts, furniture, house construction etc (Anonymous, 1988). In India, it is one of the species prioritized by National Mission of Bamboo Application (NMBA), which is popular for utilization in cottage industries as well as for construction purposes. The seed availability of this bamboo is restricted due to its long flowering cycle of 35 years (Seethalakshmi and Kumar, 1998).

Various attempts have been made in recent past to micropropagate this particular species of bamboo. Yasodha et al. (1997; 2008); Kalia et al. (2004); Islam and Rahman (2005); Negi and Saxena (2011) reported establishment of *in vitro* culture of *B. nutans* by using seed, leaf, internodal and nodal explants. However, until now, no report has been available regarding effect of various disinfecting agents, nodal positions, and seasonal variations on *in vitro* derived clones of *B. nutans*. Moreover, field performance and survivability of this species has not also been studied yet. Therefore, it is proposed to exploit the *in vitro* micropropagation technique to develop an efficient and reproducible protocol of *B. nutans*.

MATERIALS AND METHODS

Explant preparation

Commercially, important *B. nutans* was obtained from experimental farm of CSIR-North East Institute of Science and Technology, Jorhat, Assam, India (Figure 1a). Tender nodes (12 to 18 mm in length, due to variation in sizes) obtained from minor branches of 1½-year-old culms of *B. nutans* regenerated from approximately 40 year elite plants having single axillary buds were used as explants (Figure 1b to 1c). Explants were collected from the farm at the middle of every month from the same donor plant throughout the year for culture establishment. Seeds of *B. nutans* were collected from M/S Shidh Seeds Sales Corporation, Dehradun, India. After removal of leaf sheath, individual node containing axillary buds (explants without a visible bud were discarded at this point) of *B. nutans* were washed with running tap water for 10 min. Explants were then cut into 12 to 18 mm in length and node containing axillary bud was dipped in 5% (v/v) Tween 20 solution for 3 h for tissue softening followed by thorough washing under running tap water for 20 min (Figure 1d).

Establishment of *in vitro* culture

A set of 25 nodal explants was inoculated (one explant/culture vessel) each month into the MS (Murashige and Skoog, 1962) basal medium containing 1.0 mg/L of BAP (6-benzylaminopurine) for shoot induction and proliferation. The inoculated culture materials were kept in culture room, maintained at 25±2°C temperature and 60 to 70% relative humidity. All cultures were maintained under 16 h photoperiod with light intensity of 10 µmol m^{-2} S^{-1} (Cool white fluorescent light).

Effect of disinfecting agents on axenic culture establishment

Nodal explants were decontaminated with mixtures of disinfecting agents (for 5 min) prior to surface sterilization for optimal recovery of *in vitro* culture. Thereafter, surface sterilization was done with 0.1% mercuric chloride solution for 5 to 7 min and rinsed thoroughly with sterile distilled water prior to culture *in vitro* and for initial axenic establishment; cut ends of nodal explants were trimmed and placed vertically on 25 ml of culture medium in culture tubes. Numbers of nodal explants exhibiting bud-break and percentage of contaminants were recorded each day for observing efficiency of tested agents in contaminant reduction.

Effect of node positions on *in vitro* shoot culture establishment

Collected explants were cut into appropriate sizes (12 to 18 mm) due to variability of nodal explants and arranged them according to their nodal position from 1st to 11th (that is, from apex to base). After proper sterilization, explants were cultured accordingly. The bud break frequency of the nodal explants in comparison to their size and position was scored.

Influence of seasons on *in vitro* shoot culture establishment

As per the Indian noted six seasons, we have collected experimental materials in six different seasons in the middle of every month throughout the year to determine the most suitable time for culture establishment from the same donor plant in three successive years. Bud break frequencies of the axillary buds towards the six different seasons were recorded.

In vitro seed germination

After dehusking, healthy seeds of *B. nutans* were selected for culture establishment. They were given a quick rinse in 70% ethanol and then washed with 5% Tween 20 for 10 min. The seeds were further surface sterilized by treating them with 0.1% mercuric chloride for 10 min. After three washings in sterile double distilled water, the seeds were cultured on different combination of MS medium and observed their responses towards germination at 16 h light and 8 h dark condition and continuous dark condition within 15 days duration. Seed germination of *B. nutans* was carried out on both ½ strength of MS and MS basal medium. Different combination of BAP (1.0 to 5.0 mg/l) with one concentration of GA_3 (0.5 mg/L) was also tested.

In vitro shoot multiplication

Proliferated shoots (2 shoots/clump) from the axillary buds of *B. nutans* were excised and cultured into MS and B_5 (Gamborg et al., 1968) medium by supplementation of various concentration of cytokinin, auxin and GA_3 (gibberellic acid). Different concentrations (1.0 to 3.0 mg/L) of BAP, Kin (Kinetin) and 2ip (2-isopentenyladenine) with or without supplementation of GA_3 (0.5 mg/L) and NAA (0.1 and 0.5 mg/L) were also tested.

Effect of shoot clump size on shoot multiplication and growth

The *in vitro* regenerated shoots of *B. nutans* were sub-cultured every 20 to 25 days. After selecting the best treatment, clusters having more or less 2 shoots of *B. nutans* were sub-cultured into the responsive optimal media for observing their effect on shoot multiplication.

In vitro root induction

Different concentrations (0.5 to 5.0 mg/L) of auxins, that is, NAA (α-

Figure 1. a. Mother plant. **b.** Newly emerging minor secondary branch. **c.** Tender branches of *B. nutans*. **d.** Nodal explants. **e.** Axillary shoot bud initiation. **f.** Shoot initiation from shoot bud culture. **g.** Optimum shoot multiplication. **h.** Root induction. **i.** Rooted shoot. **j.** Hardening stage. **k.** Emergence of white coloured new secondary roots. **l.** Acclimatization. **m.** 1-year old plant in field condition.

naphthalene acetic acid), IBA (Indole-3-butyric acid) and IAA (Indole-3-acetic acid) were tested for the root induction frequency of *B. nutans*. Data were recorded after 30 to 35 days of culture period. The steps involved in hardening procedure of *B. nutans* were transfer of rooted shoot to MS basal liquid medium for 15 to 20 days, followed by exposing the rooted shoots in half strength of MS basal liquid medium (without sucrose) for another 15 days. After that, plantlets were kept in unsterilized filtered water for a total of 30 days that is, 15 days in culture room followed by another 15 days in ambient room temperature (28±2°C) conditions. During that stage, rooted shoots were washed thoroughly in running tap water to remove adhered agar from the roots. Caps of the flasks were also removed. Hardened plantlets of *B. nutans* were transferred to netted green house for acclimatization before transferring to the field.

Statistical analysis

The experiments were conducted in completely randomized design consisted of three replications. Experimental results were analyzed statistically using the techniques of analysis of variance for single factor experiments. The significance of the treatment means differences were tested by the procedure of Duncan's Multiple Range Test (Duncan, 1955).

RESULTS AND DISCUSSION

Morphogenetic responses of surface sterilized explants of *B. nutans* were assayed for optimization of the following factors for establishment of *in vitro* shoot culture.

Effect of different disinfecting agents on optimal recovery of *in vitro* culture

In our experiment, contamination was observed within the

Table 1. Effect of different pre-treating agents on *in vitro* culture establishment of *B. nutans.*

Treatment	Contaminant (%)	Survivability (%)
Tween 20 (5%)	85	15
Savlon (2%)	83	17
Tween 20 (5%) + Alcohol (70%)	78	22
Savlon (2%) + Alcohol (70%)	75	25
Tween 20 (5%) + Savlon (2%)	70	30
Tween 20 (5%) + Gentamicin (0.1%)	68	32
Tween 20 (5%) + Mancozeb (0.1%)	65	35
Tween 20 (5%) + Savlon (2%) + Alcohol (70%)	62	38
Tween 20 (5%) + Mancozeb (0.1%) + Gentamicin (0.1%)	58	42
Tween 20 (5%) + Mancozeb (0.1%) + Gentamicin (0.1%) + Alcohol (70%)	55	45

first 10 days in culture. Both bacterial and fungal contaminants appeared at the cut ends of the node or near the axillary buds of *B. nutans*. This fact suggests that majority of the contaminants were enclosed within the sheaths that cover the bud, and due to which disinfection agents do not reach the surface area. Sometimes contaminants appeared even after 3rd or 4th sub-cultures, when the shoot had prolific growth. Hence, various mixtures of pretreatments containing Mancozeb and Gentamicin etc were used to study their effectiveness towards *in vitro* culture establishment of this species. Among the ten different mixtures used, Tween 20 (5%) + 0.1% solution of Mancozeb (fungicide) + Gentamicin (antibiotic) + alcohol (70%) containing treatment was the best for axenic culture establishment of *B. nutans* (Table 1). In this case, 45% culture was recovered. However, single use of Tween 20 and Savlon did not show good response towards culture establishment and 85% infection was recorded. Like our study, Jimenez et al. (2006) also followed a disinfection procedure that comprised the sequential use of an alkaline detergent, a mixture of Benomyl and Agri-mycin, followed by immersion in sodium hypochlorite (1.5% w/v); but observed microbial contaminants within the first 10 days in the culture. In contrast to our work, Ramanayake and Yakandawala (1997) did not observe contamination for first 3 weeks of culture in *Dendrocalamus giganteus*.

In vitro nodal culture of *Dendrocalamus strictus* was also obstructed by microbial culture contaminants (Mascarenhas et al., 1988). Dalsaso and Guevara (1989) used different disinfection pretreatments for the explants of *Persea americana*. Ramanayake and Yakandawala (1997) used Benlate as a disinfectant for reducing the contaminants of *D. giganteus* and incorporate into the media. However, Yasodha et al. (2008) applied Streptomycin and Kanamycin as a disinfectant for *B. nutans* with 30% culture establishment. But, we had recorded maximum 45% recovery in our study on *B. nutans*. The morphogenetic competence of nodal explants of *B. nutans* was adversely affected by the phenolic exudates release from the excised explants, which caused browning of the medium and ultimately resulted in necrotic appearance of the shoots. These brown colour phenolic exudates were also released from the basal portion of the nodal explants from 4th d onwards from this species. In this case, frequent transfer to the fresh medium led to overcome this problem. Similarly, Das and Pal (2005) also recorded same type of exudates in *Bambusa balcooa* during culture initiation from nodal explants.

Effect of various node explants on *in vitro* culture initiation

It is an established fact that different parts of a plant vary in their regenerative potentiality (Evans et al., 1981; Chaturvedi, 1984). In case of *B. nutans* 5th to 7th node containing axillary bud showed maximum regeneration potentiality (Figure 1e and 1f). In this case, up to 7.0 shoot buds were initiated (Table 2). Significant difference was recorded among the 1st, 2nd, 3rd and 4th node position. No significant differences were recorded between 8th and 9th node. No bud breaking occurred beyond 9th node onwards. Hence, for further study, only selected node position, that is, 5th to 7th nodes were taken for *B. nutans*. The above result indicated that the variation of new shoot emergence may be due to size, age or other associated conditions of explants. As per report of McClure (1966), nature dormancy and breaking dormancy in buds of bamboo varied with their position in the plant, the season of the year and the species. Similarly, Saxena and Bhojwani (1993) reported the mid culm nodes of secondary branches as the best explant for axillary shoot initiation.

Influence of different seasons on *in vitro* culture establishment

The season of explant collection is a critical factor in the establishment and growth of *in vitro* cultures (Hu and Wang, 1983; Tisserat, 1985). In certain cases, the season

Table 2. Effect of various node explants on *in vitro* culture initiation of *B. nutans*.

Node position	Girth of node (cm)	Axillary bud break/explant	Shoot length (cm)
1st	0.8	2.1[f]	1.0[f]
2nd	0.9	2.9[e]	1.1[e]
3rd	1.0	3.5[d]	1.3[d]
4th	1.0	5.2[b]	1.4[cd]
5th	1.3	**6.7[a]**	**1.8[a]**
6th	1.4	**7.0[a]**	**1.8[a]**
7th	1.4	**6.9[a]**	1.6[b]
8th	1.5	4.2[c]	1.5[bc]
9th	1.5	3.7[c]	1.2[de]
10th	1.6	---	---
11th	1.6	---	---

Means are from four repeated experiments. Each treatment consisted of three replicates. Means within a row followed by the same letters are not significant at $P = 0.05$ according to DMRT.

Table 3. Effect of different seasons on *in vitro* culture establishment of *B. nutans*

Different season	Months of collection	Percentage (%) Bud break	Shoot number/explant
Winter	January–February	35.02[c]	1.93[d]
Spring	March-April	25.00[d]	3.02[c]
Summer	May-June	20.00[e]	2.60[c]
Monsoon	July-August	15.04[f]	5.50[b]
Early Autumn	September-October	**45.00[a]**	**6.30[a]**
Late Autumn	November-December	40.00[b]	5.90[a]

Means are from four repeated experiments. Each treatment consisted of three replicates. Means within a row followed by the same letters are not significant at $P = 0.05$ according to DMRT.

of explants collection is more important than the selection of a right kind of media (Chaturvedi, 1984). In *B. nutans,* maximum bud break (45%) and number of proliferating shoots (5.90) took place during early autumn (Table 3). In winter, 35.02% of bud breaking was recorded. In this season, minimum number of sprouted shoot bud was recorded for the species. During this period, phenolic exudation was more and shoot growth was recorded as slow. This result supported the findings of Sahoo and Chand (1998) and Andersone and Ievinsh (2002) in *Tridax procumbens* and *Pinus sylvestris*. In spring and summer, bud breaking and shoot growth of the species decreased. During these seasons, microbial contamination and browning of sprouted axillary buds was more. These are the major constrains for axenic establishment of culture.

In monsoon, minimum bud breaking percentage of the species was recorded. Explants collected during this season showed reduced phenolic exudation. Sprouted axillary buds were heavily contaminated in this season even after 3 to 4 weeks of culture period. The influence of seasonal rainfall pattern on the rate of axillary bud-break and fungal contamination in bamboos was reported previously by (Ramanayake and Yakandawala 1997; Saxena and Bhojwani, 1993).

In vitro seed germination

Seed germination started from the 2nd to 3rd day onwards in both 8 h dark and continuous dark condition. 30% fungal contamination was observed after 8 days culture period. Moreover, contaminants appeared together with the emergence of the radicle. This indicates the presence of endogenous microorganisms within the seed. Arce-Montoya et al. (2006) reported the presence of endogenous fungus from the seeds of *Yucca valida*. A varied germination rate and shoot growth was recorded in respect of exposure to different cultural condition. Moreover, results also varied as per the additional supplements of various hormones. Between the two cultural conditions tested for germination, maximum germination rate up to 63% was recorded from the treatment of ½ strength of MS basal, from the cultures kept in 16 h light condition (Table 4). Keeping the same treatment in total dark condition resulted lower germination percentage

Table 4. Effect of photoperiod and growth regulators on seed germination of *B. nutans*.

Basal Medium	BAP (mg L^{-1})	GA_3 (mg L^{-1})	Seed germination (%)		Number of shoot emergence		Shoot length (cm)	
			Dark	16 h light	Dark	16-h light	Dark	16 h light
½ strength of MS	-	-	**50.33^{a}**	**63.00^{a}**	0.64^{e}	0.90^{e}	**1.50^{ab}**	**1.07^{ab}**
½ strength of MS	1.0	-	47.80^{a}	45.00^{d}	1.00^{cd}	1.00^{de}	1.07^{abc}	0.80^{b}
½ strength of MS	1.0	0.5	33.33^{c}	41.33^{e}	1.12^{cd}	1.10^{de}	0.93^{abc}	0.83^{b}
½ strength of MS	2.0	-	39.67^{b}	40.33^{e}	**2.33^{abc}**	**2.00^{cd}**	0.60^{bc}	0.57^{b}
½ strength of MS	3.0	-	32.77^{d}	39.00^{f}	2.00^{abcd}	1.15^{de}	0.60^{bc}	0.37^{b}
½ strength of MS	4.0	-	30.00^{cd}	41.00^{e}	1.00^{cd}	1.12^{de}	0.60^{bc}	0.47^{b}
½ strength of MS	5.0	-	25.07^{d}	18.67^{h}	1.69^{bcd}	1.13^{de}	0.60^{bc}	0.33^{b}
MS	-	-	**49.00^{a}**	**55.00^{b}**	0.72^{d}	1.12^{de}	**1.77^{a}**	**1.30^{a}**
MS	1.0	-	45.33^{ab}	51.67^{c}	1.33^{bcd}	2.00^{cd}	1.51^{ab}	0.90^{b}
MS	1.0	0.5	31.67^{c}	46.00^{d}	1.67^{bcd}	2.67^{c}	1.19^{abc}	0.83^{b}
MS	2.0	-	14.33^{ef}	23.00^{g}	**3.33^{a}**	**6.00^{a}**	0.93^{abc}	0.40^{b}
MS	3.0	-	11.67^{f}	29.00^{f}	2.67^{ab}	4.67^{b}	0.60^{bc}	0.36^{b}
MS	4.0	-	17.67^{e}	15.67^{i}	2.00^{abcd}	2.00^{cd}	0.45^{c}	0.40^{b}
MS	5.0	-	14.77^{ef}	15.67^{i}	1.33^{bcd}	1.20^{de}	0.60^{bc}	0.30^{b}

Means are from four repeated experiments. Each treatment consisted of three replicates. Means within a row followed by the same letters are not significant at $P = 0.05$ according to DMRT. BAP = 6-Benzylaminopurine; GA_3 = Gibberellic acid; MS = Murashige and Skoog medium.

of 50.33. Treatment containing MS basal also showed satisfactory results regarding seed germination at both the cultural conditions (that is, 49.00 and 55.00, respectively). Addition of BAP (1.0 and 5.0 mg/L) to the above basal medium could not enhance seed germination. BAP (2.0 mg/L) enriched MS basal media, showed maximum number of shoot emergence (6.00) from the culture kept in 16 h light condition. However, treatment with increase concentrations of BAP (3.0 to 5.0 mg/L) resulted in gradual reduction of shoot numbers from both the basal medium as well as in both the cultural condition. Considering the shoot length, it can be said that there is a relation with the photoperiod. In this experiment, we observed maximum shoot length (1.77 cm) from the MS basal medium without any PGR and in total dark condition. In this case regenerated shoots were whitish in colour and thin. The same treatment with light condition generated healthy and normal growth with shoot length 1.07 cm. From the above study, it can be said that minimal nutritional support, that is, ½ strength of MS is enough to obtain optimal germination of *B. nutans*. To enhance multiple shoot regeneration, BAP (2.0 mg/L) can be added to the basal medium. Moreover, 16 h light condition was the best to get normal and healthy seedlings. Similar studies were conducted on seed germination of three bamboo species, viz. *Dendrocalamus membranaceus*, *D. strictus* and *B. nutans* to determine the best cultural combination. In this case, the presence of light was found to be the ideal condition for seed germination (Rawat, 2005). When the seedlings attained a growth of 6 to 7 cm, these were transferred to the MS basal liquid medium followed by soil for further growth. One-year-old seedlings of *B. nutans* showed various morphological differences in respect of their growth, shoot length, tiller numbers, leaf numbers and leaf sizes etc. Different leaf morphology, that is, either with alternate leaf pattern or opposite leaf pattern was observed. Variation in tiller numbers and shoot numbers were reflected, the heterozygous characteristics of the seed raised plants (Figure 2a, b, c, d, e, f, g, h).

In vitro shoot multiplication

Among the single concentration of BAP (1.0, 2.0, 3.0 mg/L), 1.0 mg/L BAP induced more number of shoots, with increase shoot length and leaf numbers in comparison to the other two concentrations. These shoots no longer attained its greenish colour. Therefore, single combination of BAP was not applied in further experimental work. Combined effect of BAP (0.5 and 1.0 mg/L) with NAA (0.1 and 0.5 mg/L) was also tested. Under this investigation, treatment containing BAP (0.5 mg/L) and NAA (0.1 mg/L) enhanced the maximum morphogenetic potential of *B. nutans* (Figure 1g). In this case, shoot numbers and shoot length was recorded as 11.33 and 4.40 cm, respectively. Here, shoots were greenish and healthy in nature. In this treatment, a 4 fold increase of shoot length was obtained. Similarly, combination of BAP (1.0 mg/L) with NAA (0.1 mg/L) resulted in maximum leaf numbers (18.30). In this regard, shoot length and shoot number was not encouraging with 8.33 shoot numbers and 2.83 cm shoot length. Moreover, BAP (1.0 mg/L) and NAA (0.5 mg/L) combination was

Figure 2. a. Seeds. **b.** Germinated seedlings. **c – h.** Various morphological differences obtained from *in vitro* germinated seedlings.

also not suitable for shoot regeneration resulting 6.33 shoot numbers, 2.73 cm shoot length and 10.67 leaf numbers. Addition of Kin (0.5 mg/L), BAP (1.0 mg/L) and NAA (0.1 mg/L) did not show any significant result. Likewise, single supplementation of Kin (1.0, 2.0, 3.0 mg/L each) did not enhance the shoot regeneration capacity of *B. nutans*. Addition of 2 ip alone had not affected the shoot multiplication of *B. nutans*. Moreover, GA_3 (0.5 mg/L) along with various concentration of BAP (viz. 1.0, 2.0, 3.0 mg/L) also did not show any significant result. Yasodha et al. (2008) used BAP alone and obtained only a 3 fold increase in shoot multiplication. However, Negi and Saxena (2011) obtained 3.5 fold shoot multiplication in MS liquid medium supplemented with 13.2 lM BAP, 2.32 lM Kin, and 0.98 lM indole-3-butyric acid (IBA).

When the sprouted shoots of *B. nutans* were transferred to the B_5 medium with BAP (1.0 mg/L) recorded less number of shoots (6.00) and shoots length (1.93 cm) and leaf numbers (5.33). MS basal and ½ strength of MS basal media either alone or supplemented with BAP (1.0 to 3.0 mg/L) were not suitable for shoot proliferation. Addition of GA_3 (0.5 mg/L) in combination with BAP did not show any significant result (Table 5a). Hence, the use of basal media without PGRs resulted in gradual reduction in shoot and leaf numbers and shoot length, although regeneration of shoots was recorded immediately after placing to these media. Under this study, the shoot multiplication rate could be maintained up to 6th sub-culture cycle and then gradually declined. Similar to our findings, Arya et al. (2008) also reported same rate of shoot multiplication for *Dendrocalamus asper*. Similar results was demonstrated by various workers on many bamboo species viz. in *B. balcooa* (Dutta-Mudoi and Borthakur, 2009) and *Drosera hamiltonii* (Agnihotri and Nandi, 2009; Agnihotri et al., 2009). The multiplication cycles of shoots did not involve a callus phase in this species.

Effect of shoot clump size on shoot multiplication and growth

Four shoots/clump was the best propagule size for *in vitro* shoot multiplication of *B. nutans*. In this case, maximum shoot numbers (11.40), shoot length (4.66 cm) and leaf numbers (18.60) were recorded (Table 5b). Three shoots/clump showed same significant result with the four shoots/ clump size in relation with shoot numbers and shoot length. In this case the nature of the shoots was not so satisfactory. Similarly, 5 shoots/clump also showed same result as above. In this case, leaves and shoots gradually turned pale green in colour. Similarly, 1 shoot/ clump and 2 shoots/clump were also not appropriate for shoot multiplication. It was observed that apart from optimal medium composition towards production of healthy

Table 5a. Morphogenetic response of *B. nutans* at different concentration and combination of growth regulators.

Basal medium	Kin (mg L^{-1})	2ip (mg L^{-1})	BAP (mg L^{-1})	NAA (mg L^{-1})	GA_3 (mg L^{-1})	Number of shoots	Shoot length (cm)	Number of leaves
½ MS	-	-	-	-	-	2.33^{j}	1.00^{d}	4.69^{gh}
½ MS	-	-	1.0	-	-	4.67^{defghi}	1.33^{cd}	6.67^{defg}
½ MS	-	-	1.0	-	0.5	5.00^{cdefgh}	2.83^{b}	7.33^{defg}
½ MS	-	-	2.0	-	-	4.67^{defghi}	1.00^{d}	5.68^{efg}
½ MS	-	-	2.0	-	0.5	3.67^{fghij}	1.00^{d}	4.67^{gh}
½ MS	-	-	3.0	-	-	5.33^{cdefg}	1.17^{d}	8.10^{cdef}
½ MS	-	-	3.0	-	0.5	5.33^{cdefg}	1.13^{d}	8.00^{cdef}
MS basal	-	-	-	-	-	2.33^{j}	1.03^{d}	3.39^{h}
MS	1.0	-	-	-	-	2.67^{ij}	2.23^{bcd}	7.67^{cdefg}
MS	2.0	-	-	-	-	3.33^{ghij}	2.17^{bcd}	8.12^{cdef}
MS	3.0	-	-	-	-	3.00^{hij}	1.83^{bcd}	5.67^{efgh}
MS	-	1.0	-	-	-	2.67^{ij}	1.57^{bcd}	6.34^{efgh}
MS	-	2.0	-	-	-	4.00^{efghij}	2.07^{bc}	9.67^{cd}
MS	-	3.0	-	-	-	2.67^{ij}	1.57^{bcd}	6.68^{defg}
MS	-	-	1.0	-	-	7.00^{bc}	2.67^{bc}	9.67^{cd}
MS	-	-	2.0	-	-	6.00^{cde}	1.50^{bcd}	8.00^{cdef}
MS	-	-	3.0	-	-	5.67^{cdef}	1.27^{d}	8.67^{cde}
MS	-	-	0.5	0.1	-	11.33^{a}	4.40^{a}	12.00^{b}
MS	-	-	1.0	0.1	-	8.33^{b}	2.83^{b}	18.00^{a}
MS	-	-	1.0	0.5	-	6.33^{bcd}	2.73^{b}	10.67^{bc}
MS	0.5	-	1.0	0.1	-	6.00^{cde}	2.23^{bcd}	8.67^{cde}
MS	-	-	1.0	-	0.5	4.33^{defgh}	1.13^{d}	6.33^{efgh}
MS	-	-	2.0	-	0.5	4.00^{efgh}	1.17^{d}	5.00^{fgh}
MS	-	-	3.0	-	0.5	4.00^{efgh}	1.00^{d}	3.33^{h}
B_5	-	-	1.0	-	-	6.00^{cde}	1.93^{bcd}	5.33^{fgh}

Means are from four repeated experiments. Each treatment consisted of three replicates. Means within a row followed by the same letters are not significant at $P = 0.05$ according to DMRT. BAP = 6-Benzylaminopurine; GA_3 = Gibberellic acid; MS = Murashige and Skoog medium; Kin = Kinetin; 2ip = 2-isopentenyladenine; NAA = α-Naphthalene acetic acid.

Table 5b. Effect of shoot clump size on shoot multiplication and growth of *B. nutans*.

Number of shoots/clump	Shoot number	Shoot length (cm)	Leaf number
1	4.20^{c}	3.08^{c}	6.00^{c}
2	7.40^{b}	3.68^{b}	9.20^{c}
3	10.20^{a}	4.54^{a}	14.00^{b}
4	**11.40^{a}**	**4.66^{a}**	**18.60^{a}**
5	9.80^{a}	3.32^{b}	15.60^{a}

Means are from four repeated experiments. Each treatment consisted of three replicates. Means within a row followed by the same letters are not significant at $P = 0.05$ according to DMRT.

shoots, regeneration capacity of the *in vitro* shoot was found to be dependent upon (1) size and number of shoots/clump and (2) time of sub-culturing. The shoot multiplication rate declined sharply if propagule of sub optimal size was taken for sub-culturing. Arya et al. (1999; 2002) studied in detail of the effectiveness of different propagule size on shoot multiplication of *D. asper*. According to their observation, 3 shoots/clump was the best propagule size for inducing shoot multiplication of *D. asper*.

In vitro root induction

Among the different treatments, highest adventitious rooting frequency was obtained on NAA (2.0 mg/L) supplemented medium. In this treatment, maximum root length (1.66 cm) and root numbers (2.60) was achieved after 30 to 35 days of culture (Table 6). Increased level of

Table 6. Effect of different auxins on rooting of *in vitro* shoots of *B. nutans*.

NAA (mg L^{-1})	IBA (mg L^{-1})	IAA (mg L^{-1})	Number of roots	Root length (cm)
0.50	-	-	0.96^{bc}	0.98^{bc}
1.00	-	-	1.30^{b}	1.20^{ab}
2.00	-	-	2.60^{a}	1.66^{a}
3.00	-	-	1.28^{b}	1.12^{b}
4.00	-	-	1.00^{bc}	1.05^{bc}
5.00	-	-	0.60^{c}	0.66^{c}
-	0.50	-	0.00	0.00
-	1.00	-	0.00	0.00
-	2.00	-	0.00	0.00
-	3.00	-	0.00	0.00
-	4.00	-	0.00	0.00
-	5.00	-	0.00	0.00
-	-	0.50	0.00	0.00
-	-	1.00	0.00	0.00
-	-	2.00	0.00	0.00
-	-	3.00	0.00	0.00
-	-	4.00	0.00	0.00
-	-	5.00	0.00	0.00

Means are from four repeated experiments. Each treatment consisted of three replicates. Means within a row followed by the same letters are not significant at P = 0.05 according to DMRT. NAA = α-Naphthalene acetic acid; IBA = indole-3-butyric acid; IAA = indole-3-acetic acid.

NAA (5.0 mg/L) resulted in limited root induction as well as reduction of root length and numbers. In this treatment, roots were thick and stout. Survivability percentage of these rooted shoots were also very less. The addition of NAA at 3.0 and 4.0 mg/L produced roots of a good quality, but root induction frequency was low (1.28 and 1.00). Similarly, lower concentration of NAA (0.5 and 1.0 mg/L) also resulted less number of root induction (0.96 and 1.30), respectively. In this case, the roots were thin. Treatments with IBA and IAA did not induce root induction at all. Unlike our study, Yasodha et al. (1997; 2008) obtained optimal root induction of *B. nutans* on IBA fortified medium. Hence, medium containing 2.0 mg/L NAA was the best for high frequency root induction of *B. nutans* (Figure 1h and i). During our study, we had recorded 85% rooting after 30 to 35 days of culture. Yasodha et al. (2008) recorded only 68% rooting in this bamboo species. Similarly to our study, the effect of single concentration of NAA was reported as optimal for root induction of *Berberis vulgaris, D. asper* and *D. membranaceus* (Arya et al., 2002) and *Dendrocalamus latiflorus* (Lin et al., 2007).

Hardening of *in vitro* plantlets

After root initiation, when the roots of *B. nutans* attained a length of 0.5 to 1.0 cm, then the rooted shoots were transferred to MS basal liquid medium for 15 to 20 days for both shoot and root elongation, which was followed by exposing the rooted shoots in half strength of MS basal liquid medium for another 15 days. Plantlets were then kept in unsterilized filtered water for 30 days; 15 days in culture room followed by another 15 days in ambient room temperature (28±2°C) conditions. During this phase, white-colour new secondary roots developed. In the hardening stage of *B. nutans*, we had lost 10 to 15% plantlets and 70% survivability was recorded (Table 7; Figure 1j and k). To overcome this problem, ideal shoot selection for rooting (20 to 25 days old; 1.5 to 2.5 cm in length) was the most important factor. It allowed the minimizing of the mortality rate during the hardening period.

Acclimatization and field response of tissue culture raised plantlets

The transplantation stage continues to be major bottle neck in the micro propagation of many plants (Hazarika, 2003). Plantlets that were grown *in vitro* had continuously exposed to a unique microenvironment with high level of humidity, aseptic conditions, on a medium containing ample sugar and nutrients. Thereafter, *in vitro* raised plantlets of the species should be gradually acclimatized to the environment of the poly house or green house with lower relative humidity, higher light level, septic environment that was stressful to *in vitro* raised plantlets. Successful transfer of plantlets from tissue-culture vessels

Table 7. General description of hardening stage in *B. nutans*.

Factor	Standard
General appearance	Healthy, greenish
Height of plantlets	3.0-6.0 cm
Tillers/ plantlets	2-4
Leaves/ plantlets	5-10
Visible Nodes/ plantlets	2-5
Nature of roots	Fibrous type
Number of roots	2-6 with initiation of white coloured secondary roots
Length of roots	5-10 cm

Table 8. General description of tissue culture raised *B. nutans* plantlets during acclimatization stage.

Factor	Standard (1 to 4 months old)
General appearance	Healthy, greenish
Height of plantlets	4 - 10 cm
Tillers/ plantlets	1 - 3
Leaves/ plantlets	3 - 10
Visible Nodes/ plantlets	1 - 3
Insect/ Disease Pest infestation	Less than 10%

Table 9. Performance of tissue culture raised *B. nutans* plantlets after field transfer.

Factor	Standard	
	6 months old	12 months old
General appearance	Healthy, greenish	Healthy, greenish
Height of Plantlets	15-20 cm	22-25 cm
Tillers/ plantlets	1- 5	1-7
Leaves/ plantlets	10 -15	20-30
Visible Nodes/ plantlets	2 - 6	6-8
Insect/ Disease Pest infestation	Less than 10%	Less than 10%

to the ambient *ex vitro* condition can determine the significance of any micro propagation system.

In this study, micro propagated plantlets of *B. nutans* were potted in polythene sleeves containing 1:1:2 soil : sand : cow-dung mixture and kept in netted poly house for acclimatization process. Plantlets were healthy and greenish and grew well in poly house. In this stage, we had recorded 80% survivability in *B. nutans* and morphological characteristics were satisfactory (Table 8; Figure 1l). Satisfactory results obtained here in the transfer and acclimatization could be related to the easy adaptation of the bamboo in marginal ecological conditions (Crouzet, 1981). After acclimatization, *in vitro* raised plantlets of *B. nutans* were transferred to the field condition. In field condition, 95% survivability was recorded from *in vitro* derived plantlets evaluated after two years old *B. nutans* plantlets (Table 9; Figure 1m). In this case, field performance experiment of *B. nutans* was conducted with 6 to 24 months old plants. Micro propagated plantlets of studied bamboo species recorded well morphological characteristics in field condition. No morphological variation was observed in the species. Plantlets were grown very uniformly.

Gupta et al. (1991) also recorded positive response of tissue culture raised plantlets, that is, mainly forestry species in field condition. Similarly, Wei and Tien (1995) successfully transferred *in vitro* regenerated plants of *Bambusa beecheyana* Munro var *beecheyana* into the field.

Conclusion

The present study describes an effective regeneration and multiplication protocol for *in vitro* propagation of *B.*

nutans. Micropropagated plantlets and seedlings of this species showed considerable differences during hardening stage. As seedling raised plants proved the heterogeneous characters of the seeds; hence, *in vitro* raised shoots of *B. nutans* could be effectively used in maintaining the clonal fidelity of elite genotypes. High multiplication efficiency, good rooting, easy establishment in the soil and normal growth performance of micropropagated plants, as reported in this study, are features necessary for the adoption of *in vitro* propagation technology for large scale multiplication of this species. Thus, standardized protocol of *B. nutans* can be said as easy to raise, economic to adopt and convenient to transport, thus serving commercial interest.

Conflict of Interests

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

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REFERENCES

Agnihotri RK, Mishra J, Nandi SK (2009). Improved *in vitro* shoot multiplication and rooting of *Dendrocalamushamiltonii* Neeset Arn. Ex Munro: production of genetically uniform plants and field evaluation, Acta Physiol. Plant. 31:961-967.

Agnihotri RK, Nandi SK (2009).*In vitro* shoot cut: A high frequency multiplication and rooting method in the bamboo *Dendrocalamushamiltonii*. Biotechnology 8:259-263.

Anantachote A (1987). Flowering and seed characteristics of bamboos in Thailand in recent research on Bamboos (IDRC, Canada). pp. 33-44.

Andersone U,Ievinsh G (2002). Changes of morphogenic competence in mature *Pinussylvestris* L. *in vitro*. Ann. Bot. 99:293-298.

Anonymous (1988).The wealth of India. Raw materials, Vol. 2B (Publication and information Directorate, CSIR, New Delhi).

Arce-Montoya M, Rodriguez-Alvarez M, Hernandez-Gonzalez JA, Robert ML (2006). Micropropagation and field performance of *Yucca valida*. Plant Cell Rep. 25:777-783.

Arya S, Satsangi R, Arya ID (2002). Rapid mass multiplication of edible bamboo *Dendrocalamusasper*. J. Sustain. For. 14:103-109.

Arya S, Satsangi R, Arya ID (2008).Direct regeneration of shoots from immature inflorescences in *Dendrocalamusasper* (edible bamboo) leading to mass propagation. Bamboo Sci. Cult. 21:14-20.

Arya S, Sharma S, Kaur R, Arya ID (1999).Micropropagation of *Dendrocalamusasper* by shoot proliferation using seeds. Plant Cell Rep. 18:879-882.

Banik RL (1987). Techniques of bamboo propagation with special reference to prerooted and pre-rhizomed branch cuttings and tissue culture. In: AN Rao, G Dhanarajan, CB Sastry(eds) Recent Research on Bamboos, Proc. Int. Natl. Bamboo Workshop, Hongzhou Peoples of China and IRDC, Canada. pp. 160-169.

Chaturvedi HC (1984). Some aspects of morphogenesis in rapid multiplication and germplasm preservation of economic plants through tissue culture. In: PC Dutta (eds) Proceedings of National Symposium on Applied Biotechnology of Medicinal, Aromatic and Timber Yielding Plants, Calcutta University, Calcutta. pp. 179-188.

Crouzet Y (1981). Les bambous (INRA, PARIS). p. 95.

Dalsaso L, Guevara E (1989).Multiplicacio´n clonal *in vitro*delaguacate (*Perseaamericana*) cv. 'Fuerte'. Agron. Costarric. 13:61-71.

Das M, Pal A (2005).*In vitro* regeneration of *Bambusabalcooa*Roxb.: factors affecting changes of morphogenetic competence in the axillary buds. Plant Cell Tissue Organ Cult. 81:109-112.

Duncan DB (1955). Multiple ranges and multiple F test. Biometrics. 11:1-42.

Dutta Mudoi K, Borthakur M (2009). *In vitro* micropropagation of *Bambusa balcooa* Roxb. through nodal explants from field-grown culms and scope for upscaling. Curr. Sci. 7: 962-966.

Evans DA, Elgi JE, Kut SA, Sharp WR, Flick CK (1981).*In vitro* regeneration of the ornamental tobacco *Nicotiniaalata*. Hortic. Sci. 16: 425.

Gamborg OL, Miller RA, Ojima K (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50:151-158.

Gupta PK, Timmis R, Mascarenhas AF (1991). Field performance of micropropagated forestry species. *In Vitro* Cell Dev. Biol. Plant 27:159-164.

Hazarika BN (2003). Acclimatization of tissue-cultured plants. Curr. Sci. 85:1704-1712.

Hu CY, Wang PJ (1983). Meristem, shoot tip and bud culture. In: DA Evans, WR Sharp, PV Ammirato, Y Yamada (eds)Handbook of Plant Cell Culture, MacMillan Publishing Company, New York. pp. 177-227.

Islam SAMN, Rahman MM (2005). Micro-cloning in commercially important six bamboo species for mass propagation and at a large scale. Plant Tissue Cult. Biotechnol. 15:103-111.

Jimenez VM, Castillo J, Tavares E, Guevara E, Montiel M (2006).*In vitro* propagation of the neotropical giant bamboo, *Guaduaangustifolia* Kunth, through axillary shoot proliferation. Plant Cell Tissue Organ Cult. 86:389-395.

Kalia S, Kalia RK, Sharma SK (2004). *In vitro* regeneration of an indigenous bamboo (*Bambusanutans*) from internode and leaf explants. J Bamboo Rattan 3:217-228.

Lin CS, Liang CJ, Hsaio HW, Lin MJ, Chang WC (2007).*In vitro* flowering of green and albino *Dendrocalamuslatiflorus*. New For. 34:177-186.

Mascarenhas AF, Nadgir AC, Thengane SR, Phadke CH, Khuspe SS, Shirgurkar MV, Parasharami VA, Nadgauda RS (1988). Potential application of tissue culture for propagation of *Dendrocalamusstrictus.* In:IVR Rao, R Gnanaharan, CB Shastry(eds) Bamboos, Current Research, Proc. Intl. Bamboo Workshop, Cochin, India. pp. 159-166.

McClure FA (1966).The Bamboos: A Fresh Perspective. Harvard University Press, Cambridge, Mass, USA.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473-497.

Negi D, Saxena S (2011).*In vitro* propagation of *Bambusanutans* Wall. Ex Munro through axillary shoot proliferation. Plant Biotechnol Res. 5: 35-43.

Ramanayake SMSD, Yakandawala K (1997).Micropropagation of the giant bamboo (*Dendrocalamusgiganteus* Munro) from nodal explants of field grown culms. Plant Sci. 129:213-223.

Rawat MMS (2005). Optimum conditions for testing germination of bamboo seeds. J. Bamboo Rattan 4:3-11.

Sahoo Y, Chand PK (1998).*In vitro* multiplication of a medicinal herb, *Tridaxprocumbens* L. (Mexican Daisy, coatbuttons): influence of explanting season, growth regulator synergy, culture passage and planting substrate. Phytomorphology 48:195-205.

Saxena S, Bhojwani SS (1993).*In vitro* clonal multiplication of four year old plants of the *Dendrocalamuslongispathus*Kurz. *In Vitro Cell.* Dev. Biol. Plant 290:135-142.

Seethalakshmi KK, Muktesh Kumar MS (1998).Bamboos of India – a Compendium. Bamboo Information Centre – India, Kerala Forest Research Institute, Peechi and International Network for Bamboo and Rattan, Beijing. Eindhoven, New Delhi.

Tisserat B (1985). Embryogenesis, organogenesis and plant regeneration. In: RA Dixon (eds)Plant cell culture - a practical approach,IRL Press, Washington D. C. pp. 79-105.

Wei CC, Tien HL (1995). Somatic embryogenesis and plant regeneration from roots of bamboo (*Bambusabeecheyana* Munro var*beecheyana*). J. Plant Physiol.145:535-538.

Yasodha R, Kamala S, Ananda Kumar SP, Durai Kumar P, Kalaiarasi K (2008). Effect of glucose on *in vitro* rooting of mature plants of *Bambusanutans.* Sci. Hortic. 116:113-116.

Yasodha R, Sumathi R, Malliga P, Gurumurthi K (1997). Genetic enhancement and mass production of quality propagules of *Bambusanutans* and *Dendrocalamusmembranaceous*. Indian For. 123:303-306.

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Chemical characterization and local dispersion of slag generated by a lead recovery plant in Central Mexico

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A toxic waste "slag" generated in a lead (Pb) recovery plant was characterized and its local "slag" dispersion was monitored. The major constituents of the "slag", in increasing weight percent, were CaO (1.5), PbO (3.4), SiO_2 (5.2), Na_2O (17.8), SO_3 (27.5) and Fe_2O_3 (40.7). The "slag" was alkaline with a pH of 10 and an electrical conductivity of 606 dS m^{-1}. Tests for Pb leaching indicated that, in a moderately acid environment, as much as 8 mg Pb L^{-1} may be washed out of the "slag"; there are amounts that are well above the limits set by Mexican legislation. The highest concentration of Pb in soil surrounding the recycling plant (447 mg Pb kg^{-1}) was found 50 m southwest of the recovery plant. Due to its high Pb level and easy dispersion into the environment, the health of human settlements in the area could be at risk.

Key words: Toxic waste, alkaline material, Pb leaching.

INTRODUCTION

According to Mexican Official Norm (NOM-052-SEMARNAT-2005), exhausted lead-acid automobile batteries are considered hazardous wastes, due to their high lead (Pb) content. In Mexico, 95% of the acid batteries discarded are recycled, and the efficiency of Pb recovery is about 98% according to the Instituto Nacional de Ecología (INE, 2000). A typical process for recycling Pb from acid batteries includes their rupture, draining of the acid, separation of the components containing Pb and its recovery by smelting (Faé et al., 2011). During the smelting process, a solid material called "slag" is generated. It contains a high concentration of Pb, among other toxic elements (Coya et al., 2000; Penpolcharoen, 2005).

"Slag" and the fumes from the chimneys of the smelting furnace thus represent a risk to human health and the environment (Andrade Lima and Bernardez, 2011). Pb causes a wide range of biochemical, physiological and behavioral dysfunctions. The mechanisms of Pb toxicity to living organisms at the molecular level are caused by covalent binding to proteins, oxidative damage and interference with divalent cation specific sites, such as

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Table 1. Components of the Pb"slag" determined by X-ray fluorescence.

Compound	Weight (%)
TiO_2	0.056
P_2O_5	0.084
V_2O_5	0.104
ZnO	0.110
K_2O	0.121
CrO_3	0.142
Sb_2O_3	0.158
MgO	0.195
As_2O_3	0.246
MnO	0.255
CuO	0.278
Cl	0.317
Al_2O_3	0.423
BaO	0.554
SnO_2	0.890
CaO	1.460
PbO	3.430
SiO_2	5.180
Na_2O	17.840
SO_3	27.450
Fe_2O_3	40.740

those recognized by Ca^{++} (Quintanar-Escorza et al., 2007). It also causes damage to DNA (Yáñez et al., 2003). In addition, high Pb levels in the atmosphere generate a negative impact on human health, plants, animals and microorganisms (Akmal et al., 2005; Zhuang et al., 2009). As a result of excessive intake of Pb by plants and animals this element can reach the food chain, which becomes a potential risk to human health (Zhuang et al., 2009).

Farago et al. (1999) reported Pb levels that ranged between 792 to 39 mg Pb kg^{-1}in top soils (0-5 cm) at distances of 0.5 and 3.5 km, respectively, from a Pb recycling plant in southern Sweden. In fact, in a study conducted in north Mexico, it was found that, in human populations located at 5 and 0.9 km from a smelter, the average Pb level in blood (ABL) was 7.8 and 21.8 μg dL^{-1}, respectively (García et al., 1999). Regarding to the Pb recovery plant under study, which generates 1500-2000 t of "slag" yr^{-1}, previous reports indicated that workers present a high Pb ABL of 74.4 ± 21.9 μg dL^{-1}(Quintanar-Escorza et al., 2007).

The objectives of this study were to chemically characterize the industrial "slag" generated by a Pb recovery plant and to monitor the local dispersion of "slag" in the plant surroundings soils.

MATERIALS AND METHODS

The study site was a Pb recovery plant located in the State of Guanajuato, Mexico at 101º 41' 00"west, 21º 07' 22" north and an altitude of 1798 m. The smelter is dedicated to the recovering of Pb from exhausted automotive and industrial batteries. Ten samples of approximately 1 kg each were taken from the "slag" heaps of the Pb-recovery plant. Sampling was conducted at different points of the deposit; samples were homogenized and a representative subsample of 1 kg was taken for further analysis.

Several analytical methods were used to evaluate the chemical composition of the "slag", including wavelength dispersive X-ray fluorescence spectrometry (WDXRF) and energy dispersive spectrometry coupled to scanning electron microscopy (SEM-EDS) (JEOL JSM model 5910LV-27, JEOL Ltd.1-2, Musashino 3-chome, Akishima, Tokyo 196-8558, Japan). These methods were used to examine the microstructure and the elemental composition of the "slag". Both types of analyses were performed on pulverized (0.8 mm) samples of "slag". The detection limit for elements using the SEM-EDS method is 0.25 weight percent (wt%) and for this reason SEM-EDS was complemented by inductively coupled plasma atomic emission spectrometry (ICP-AES) for the analyses of toxic metals present at concentrations lower than 0.25%. Three samples of "slag", each of 1 g, were digested with hydrochloric, nitric, perchloric and hydrofluoric acids (Andrade Lima and Bernardez, 2011).

The methods used here to determine the toxicity of hazardous wastes were developed by the Environmental Protection Agency of the United States of America (USEPA). They include the Toxicity Characteristic Leaching Procedure (TCLP) which was employed to determine the mobility of the metals in the "slag". Extraction tests were carried out by mixing 10 g of "slag" with acetate buffer at a ratio of 1:20 w/v (pH 4.95 ± 0.05). After stirring for 18 h at 30 ± 2 rpm at a temperature of 25 ± 2°C (Andrade Lima and Bernardez, 2011) and the Synthetic Test Precipitation Leaching Procedure (SPLP) was applied to simulate the extraction of solutes leached out by acid rain. Another extraction test was performed by exposing 10 g of "slag" to a weak solution of nitric acid/sulfuric acid (pH 5 ± 0.1) at a ratio of 1:20 w/v. Again, stirring was maintained for 18 h at 30 ± 2 rpm at a temperature of 25 ± 2°C (Andrade Lima and Bernardez, 2011). Extraction using $Ca(NO_3)_2$ to determine available Pb was also performed (Tao et al., 2006). Soluble anions (carbonates, CO_3^{-2}; bicarbonates, HCO_3^- and sulfates, SO_4^{-2}) were determined in the extract using the AS-20 method. Electrical conductivity (EC) and pH of the "slag" were determined by AS-18 and AS-02 methods, respectively (NOM-021-SEMARNAT-2000).

Samples of soil (0-30 cm depth) were collected at 14 different locations (north, south, east, west, south-west and north-west) at distances ranging from 50 to 300 m away from the recycling plant. Soil samples were air dried and stored in polyethylene bags until analysis. The pH was determined in an aqueous solution by the AS-02 method, while the EC was determined according to the AS-18 procedure. The organic matter content (OM) was analyzed by the AS-07 method, soil texture by the method AS-09 using the Bouyoucos procedure and the cation exchange capacity (CEC) by the method AS-12. All established methods are described in NOM-021-SEMARNAT-2000. Soil concentrations of Ca, Fe, Na and Pb were determined following the ICP-AES protocol. The distribution of Pb around the recycling plant was represented by an iso-curve map constructed using the program Surfer version 10.

RESULTS AND DISCUSSION

The major compounds found in "slag" were: CaO, PbO, SiO_2, Na_2O, SO_3 and Fe_2O_3, at wt% of 1.5, 3.4, 5.2, 17.8, 27.5 and 40.7, respectively (Table 1); similar values have been reported in other studies (Penpolcharoen, 2005; Andrade Lima and Bernardez, 2011). Table 2 summarizes the elemental analysis carried out by SEM-EDS and ICP-AES. The main elements in the metal fraction corresponded to 23.3, 12.6, 3.4%, Fe, Na and Pb, respectively; similar proportions were reported by Faé et al. (2011). Fe and Na are contained in the $NaHCO_3$, Fe filings and mineral coal that are added to the molten during the melting

Table 2. Elemental composition of the Pb "slag".

Element	Weight (%)
Al	0.38 ± 0.15[a]
As	0.52 ± 0.11[a]
C	7.19 ± 0.69[a]
Ca	1.57 ± 0.06[a]
Fe	23.28 ± 0.43[a]
Na	12.56 ± 0.38[a]
O	37.19 ± 0.40[a]
Pb	3.36 ± 0.29[a]
S	10.80 ± 0.16[a]
Si	2.78 ± 0.05[a]
Cd	0.04 ± 0.02[b]
Co	0.01 ± 0.01[b]
Ni	0.02 ± 0.03[b]

[a]SEM-EDS and [b]ICP-AES methods.

process (Penpolcharoen, 2005; Faé et al., 2011). The high O content (37.2%) suggests that a large proportion of these metals are present as oxides (Penpolcharoen, 2005; Andrade Lima and Bernardez, 2011).The concentration of Pb in the "slag" was 29 and 51 times higher than the maximum values allowed by Mexican legislation for agricultural soils and industrial zones, which are 400 and 700 mg kg^{-1}, respectively (NOM-147-SEMARNAT/SSA1-2004).The "slag" also contains the toxic trace elements As,Cd, Co and Ni, as shown by SEM-EDS and ICP-AES (Table 2). Only As was above the limits allowed by the Mexican legislation (NOM-147-SEMARNAT/SSA1-2004). In a study conducted in the municipality of Villa de la Paz, San Luis Potosi, Mexico levels of Pb and As in surface soil were 400 and 100 mg kg^{-1}, respectively. Here, the infant population suffers severe DNA damage due to high levels of these elements in the body (Yáñez et al., 2003). However, the levels of Pb and As in the "slag" are higher than those reported in this study, which highlights the danger posed to the population that is exposed to this pollutant.

Microscopic observation shows that the "slag" resembles a newly bituminized road surface containing prominent black grains amongst a mass of otherwise mostly homogenous material (Figure 1a). A special feature of the "slag" is the so called "white zones" that, continuing with the above analogy with a macadamized surface, resemble quartz grains. When enlarged, it is apparent that these "bright spots" possess defined structures (Figure 1b). An EDS spectrum of a "white zone"* (Figure 1b) showed high signal intensities for Fe, Pb, S, Ca, Na, O, C and As but low signals for Si and Al (Figure 1c). High signal intensities correspond to elevated concentrations of elements with large atomic numbers and, thus, the lighter areas represent elevated levels of Pb and other metals with high electron densities. The EDS spectra taken by Andrade Lima and Bernardez (2011) of larger "white spots" of "slag" indicated similar compositions to those found here.

In aqueous solution, the "slag" is alkaline, with a high EC, containing large concentrations of CO_3^{-2} but relatively low amounts of HCO_3^- and SO_4^{-2} (Table 3). Faé et al. (2011) report that freshly processed "slag" has a high pH, about 13.2 but this value decreases with "age" and stabilizes at about pH 11.2. In itself, this high level of alkalinity is sufficient to classify "slag" as toxic (Coya et al., 2000). Nevertheless, Pb leaching experiments showed (Table 4), that in a slightly acid environment, the "slag" is stable in the short term since the concentrations of Pb (4 mg L^{-1}) in the TCLP extract were below the permissible value (5 mg L^{-1}) established by Mexican legislation (NOM-052-SEMARNAT-2005). In contrast, the Pb concentration in the SPLP extract was 8 mg L^{-1} which exceeds the limits permitted suggesting that, under certain conditions, leaching from the "slag" can be an environmental hazard. Under normal conditions, the concentration of available Pb was relatively low; suggesting that ion exchange in the "slag" does not pose a large threat in the short term. However, it is difficult to predict the risks that may occur over the years (Coya et al., 2000; Faé et al., 2011).

The highest level of Pb pollution occurred southwest of the recovery plant (Table 5). Soil sampled along this transect was slightly alkaline (pH 7.5) and its EC ranged from 0.6 to 0.4 dS m^{-1} at 50 and 300 m, respectively. More salts were thus present in soil near the Pb recovery plant, probably because of dispersion of "slag" particles from the factory. Soil OM content was variable, with values of 5.3, 2.0 and 3.2% at 50,150 and 300 m, respectively, which coincided with the vegetation density. Since the soil was asandy to silty loam (NOM-021-SEMARNAT-2000), clay and OM would have the greatest influence on the CEC (Cala and Kunimine, 2003). The concentrations of Pb in the soil decreased with increasing distance from the factory, from 447 to 36 mg Pb kg^{-1} at 50 and 300 m, respectively (Table 6). In contrast, the levels of Ca and Na were relatively constant along the transect, but Fe levels mimicked those of Pb (Table 6). Similar situations have been reported by Farago et al. (1999) and Cala and Kunimine (2003). The reference value of Pb concentration in soils free of pollutants is approximately 13 mg kg^{-1} (Castillo et al., 2005). On this basis, Pb-contaminated soils have accumulated 34 fold more Pb than normal soils. Even soil samples collected 300 m from the plant exceeded reference values by 2.8 fold. Obviously, the distributions of Ca, Fe and Na do not pose the same threat as Pb to the environment.

In this particular area, dispersion of pollutants occurs in a predominantly southwest direction which coincides with the direction of the prevailing winds (Figure 2). This suggests that the population at risk is also located southwest from the recovery plant and the closest community is only about 0.5 km from the factory. In fact, workers from the plant under study already present high blood Pb concentrations (Quintanar-Escorza et al., 2007), indicating the need to regulate human settlements in areas surrounding the Pb recovery plant under study and/or to impose strict environmental standards on Pb smelters.

Alternative treatments, such as phytoremediation, have been reported as successful strategies to attenuate Pb contamination in soil (Trezena de Araújo and do Nascimento, 2010) and could be used for the recovery of

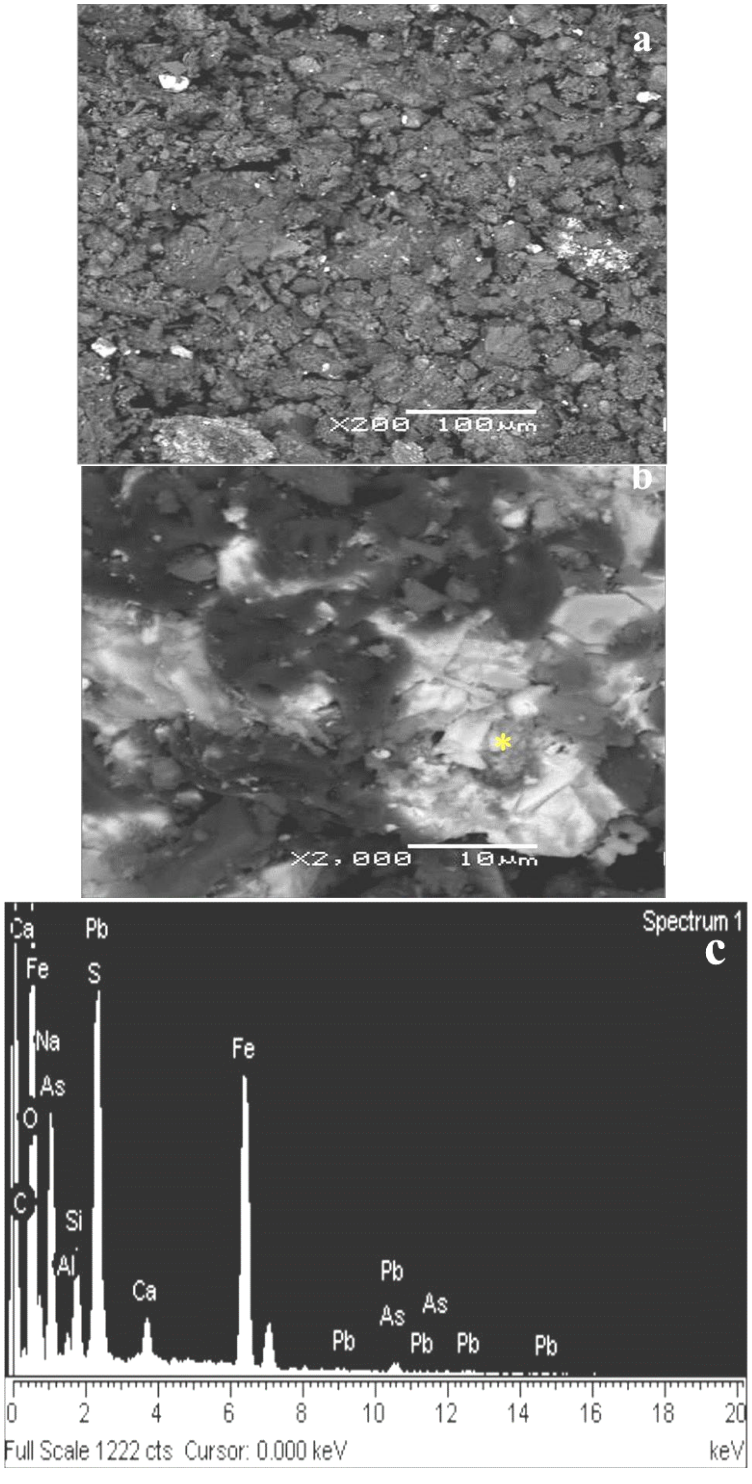

Figure 1. a-c Scanning electron microscopeimages of a Pb“slag” sample. (a) Magnified 200 times. (b) Magnified 2,000 times. Bars represent 100 µm and 10 µm, respectively. (c) EDS spectrum of a “white zone”* from Figure 1b.

areas adjacent to Pb-recovery plants.

Conclusions

High levels of PbO (3.4%), high pH (10) and increased EC (600 dS m^{-1}) in the “slag” are sufficient to consider this industrial waste as hazardous. In a moderately acid environment, as much as 8 mg Pb L^{-1} may be leached from the slag. This amount is well above the limits set by the Mexican legislation.

Hazardous concentrations of Pb, in the soil around the Pb recovery plant were found in the southwest area. The

Table 3. Chemical characteristics of Pb "slag".

Property	Content
pH	10.1± 0.02
EC (dS m^{-1})	605.6± 4.04
Anions (mmol L^{-1}):	
CO_3^{-2}	1642.0 ± 8.48
HCO_3^{-}	59.0 ± 9.89
SO_4^{-2}	183.9 ±0.020

Table 4. Concentration of lead leached out of the Pb "slag" in leaching tests.

Test	Pb (mg L^{-1})
TCLP	4.0 ± 0.003
SPLP	8.0 ± 0.010
Available Pb	4.3 ± 0.020

Table 5. Soils properties southwest of the Pb recovery plant.

Distance (m)	pH	EC (dS m^{-1})	OM	Sand	Silt	Clay	CEC (cmol kg^{-1})
				(%)			
50	7.2	0.6	5.3	52.9	36.6	10.5	30.1
150	7.5	0.5	2.0	36.9	50.6	12.5	45.9
300	7.7	0.4	3.2	36.9	50.6	12.5	43.1

Table 6. Total concentrations of metals in soil samples taken at three distances from the Pb recovery plant.

Direction	Distance (m)	Pb	Na	Ca	Fe
		(mg kg^{-1})			
Southwest	50	447	427	8700	8200
	150	149	423	12300	10400
	300	36	521	8000	4000

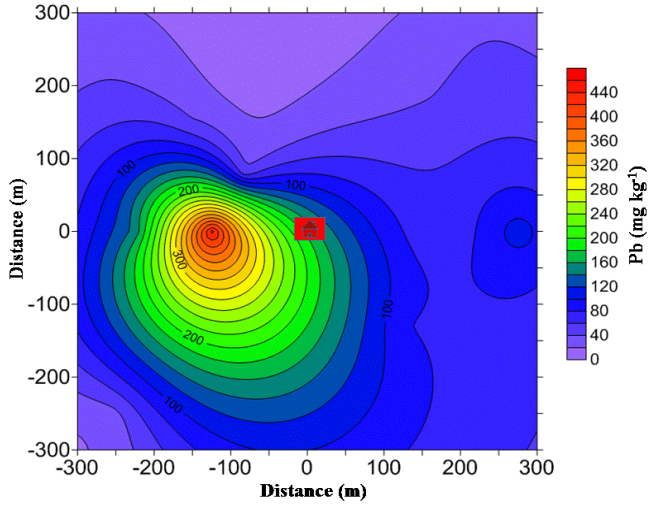

Figure 2. Superficial distribution of lead in a perimeter of 300 m around of the Pb recovery plant. Isocurves show the Pb concentration in soil. The coordinate (0, 0) indicates the location of the slag depositin the Pb recovery plant.

concentrations of Pb in soils decreased with increasing distance from the plant, being of 447, 149 and 36 mg Pb kg^{-1} at 50, 150 and 300 m, respectively. Due to these elevated Pb levels and to their easy dispersion into the environment, the health of human settlements in the area could be at risk.

Conflict of Interests

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

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REFERENCES

Akmal M, Janming X, Zhaojun L, Haizhen W, Huaiying Y (2005). Effects of lead and cadmiumnitrate on biomass and substrate utilization pattern of soil microbial communities. Chemosphere. 60:508-14.

Andrade Lima LR, Bernardez LA (2011). Characterization of the lead smelter slag in Santo Amaro, Bahia, Brazil. J. Hazard. Mater. 189:692-699.

Cala V, Kunimine Y (2003). Distribución de plomo en suelos contaminados en el entorno de una planta de reciclaje de baterías ácidas. Rev. Int. Contam. Ambie. 19:109-115.

Castillo F, Roldan M, Blasco R, Huertas M, Caballero F, Moreno C, Luque M (2005). Biotecnología ambiental. Editorial TÉBAR, Madrid, España.

Coya B, Marañon E, Sastre H (2000). Ecotoxicity assessment of slag generated in the process of recycling lead from waste batteries. Resour. Conserv. Recy. 29:291-300.

Faé G, Furlanetto T, Wada K (2011). Reduction in toxicity and generation of slag in secondary lead process. J. Clean. Prod. 19:1096-1103.

Farago M, Thornton I, White N, Tell I, Martensson M (1999). Environmental impacts of a secondary lead smelter in Landskrona, southern Sweden. Environ. Geochem. Hlth. 21:67-82.

García Vargas GG, Rubio Andrade M, del Razo Jiménez LM, Borja Aburto V, Vera Aguilar E, Cebrián García M (1999). Lead exposure in children living in a smelter community in Region Lagunera, México.J. Toxicol. Env. Heal.62: 417-419.

Instituto Nacional de Ecología (2000). Los acumuladores usados pueden dar mucha batería. In: Manual de Residuos. INE. México, DF.

Penpolcharoen M (2005). Utilization of secondary lead slag as construction material. Cement Concrete. Res. 35:1050-1055.

Quintanar-Escorza MA, González-Martínez MT, Navarro L, Maldonado M, Arévalo B, Calderón-Salinas JV (2007). Intracellular free calcium concentration and calcium transport in human erythrocytes of lead-exposed workers. Toxicol. Appl. Pharm. 220:1-8.

SEMARNAT (2005). Que establece los procedimientos de identificación, clasificación de los residuos peligrosos. Norma Oficial Mexicana 052. México, DF.

SEMARNAT (2004). Que establece criterios para determinar las concentraciones de remediación de suelos contaminados. Norma Oficial Mexicana 147. México, DF.

SEMARNAT (2000). Que establece las especificaciones de fertilidad, salinidad y clasificación de suelos, estudio, muestreo y análisis. Norma Oficial Mexicana 021. México, DF.

Tao J, Tian B, Wang H, Basta N, Schrode J, Casillas M (2006). Assessing availability, phytotoxicity and bioaccumulation of lead to ryegrass and millet based on 0.1 mol L^{-1} $Ca(NO_2)_3$ extractions. J. Environ. Sci. 18: 958-963.

Trezena de Araújo JDC, Araújo do Nascimento CW (2010). Phyto-extraction of lead from soil from a battery recycling site: The use of citric acid and NTA. Water Air Soil Pollut. 211: 113-120

Yáñez L, García-Nieto E, Rojas E, Carrizales L, Mejía J, Calderón J, Razo I, Díaz-Barriga F (2003). DNA damage in blood cells from children exposed to arsenic and lead in a mining area. Environ. Res. 93:231-40.

Zhuang P, McBride MB, Xia H, Li N, Li Z (2009). Health risk from heavy metals via comsuption of food crops in the vicinity of Dabaoshan mine, South China. Sci. Total Environ. 407:1551-1561.

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Assessment of the phytoremediation potential of *Panicum maximum* (guinea grass) for selected heavy metal removal from contaminated soils

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Non-vascular plants have potential for rapid uptake of metals, but are rarely used for phytoremediation because of their short life cycle. This property can however be advantageously used in a number of metal removal cycles within a short time. The selection of promising plants is critical to success of phytoremediation. The potential for heavy metal uptake by *Panicum maximum* a non-vascular plant was investigated using pot plant experiments. Seventy-two (72) pots of 7 L capacity were each filled with 5 kg of mixed soil collected from the Fadama (fertile soil) and College of Animal Sciences and Livestock Production farms (less fertile) of the University of Agriculture Abeokuta, Ogun State Nigeria. The pots were divided into six groups of twelve pots each. Each of the group of 12 pots was further divided into three groups of four pots each, in which *P. maximum* were planted. Five of the six groups were treated with 20, 50, 75, 100 and 120 ppm each of Pb^{2+}, Cr^{3+} and Cd^{2+} and the sixth served as control. Levels of Pb^{2+} decreased from 1.40 to 1.05 µg/g and 1.57 to 1.30 µg/g in soils treated with 20 and 120 ppm of Pb^{2+}, respectively. Generally there was increase in Pb^{2+} uptake by the different tissues of *P. maximum* ranging between 0.21 to 0.38 µg/g, 0.18 to 0.30 µg/g, and 0.09 to 0.18 µg/g in root, stem and foliage, respectively. Corresponding bioaccumulation factors (BAF) ranged between 0.21 to 0.45, 0.17 to 0.35 and 0.08 to 0.21. Metal uptake followed similar trends for Cr^{3+} and Cd^{2+} in plant tissues. The BAF values for Cr^{3+} ranged between 0.31 to 0.69 in root, 0.17 to 0.52 in stem and 0.07 to 0.34 in foliage. Similar values for Cd^{2+} were 0.14 to 0.53, 0.10 to 0.44 and 0.05 to 0.37. Accumulation of heavy metals in *P. maximum* ranged from 13 to 45%, Pb^{2+}; 13 to 65%, Cr^{3+} and 11 to 52%, Cd^{2+} of the soil concentration level with tissue abundance decreasing in the order $Cr^{3+} > Pb^{2+} > Cd^{2+}$. Furthermore, the concentration of metals in *P. maximum* tissues decreased in the order root > stem > foliage. The phytoremediation of Pb^{2+}, Cr^{3+} and Cd^{2+}contaminated soils with *P. maximum* seems to be promising under the conditions of the experiment. Obvious signs of phyto-toxicity however appeared in plants exposed to 120 ppm Pb^{2+} and Cd^{2+} at day twenty-three, suggesting that *P. maximum* may be a moderate metal accumulator.

Key words: phytoremediation, heavy metals, uptake, tissues, accumulator.

INTRODUCTION

Phytoremediation is a biological technique used in removing contaminants from contaminated soil, water or air using green plants, except for phytostabilization. The contaminants may include metals, pesticides, solvents, explosives, crude oil and its derivatives. The technique is relatively cost-effective compared to other techniques

such as metal electro-osmosis and excavation/reburial and incineration (Davies et al., 2002; Li et al., 2004). It is also environmentally friendly with significant aesthetic improvement on contaminated soils (Chen et al., 2002; Lyubun et al., 2002; Fayiaga et al., 2004) with less external input from man.

The use of phytoremediation to restore contaminated soils of abandoned metal-mine working and to reduce the impact of polychlorinated biphenyls (PCBs) from dumps and mitigation of contamination in on-going coal mine discharges has been reported (Li, 2005; Azevedo et al., 2005; Sizova et al., 2006). The efficiency and time to effect clean up by phytoremediation is a function of the plant type and population on contaminated site, concentration of pollutants and extent of pollution, soil class and prevailing soil condition which varies with locations. Soil pH, dissolved organic carbon (DOC) and the spatial distribution of electrical conductivity (EC) may however suggest ways to manage any metal loaded field area (Hattab et al., 2013). The United State Environmental Protection Agency (EPA) (2000) reported that, 'in the process of phytoremediation, plants may have to be replaced if they are destroyed by bad weather or animals, which add time to the cleanup'. Often it takes many years to clean up a site using phytoremediation procedures.

The selection of promising plants is an important approach to successful phytoremediation. Plants used for phytoremediation clean up procedures can range from those with natural ability, moderate accumulator to hyper-accumulator or those that degrade or render harmless contaminant in soils, water and air (Hemen, 2007). Various plants have been used differently for different pollutant types. Such hyper-accumulator plants reported include mustard plants, alpine, pennycress, kenaf and pigwood which were used on toxic wastes sites. Others include ladder fern *Pteris vittata* which accumulate arsenic (As) even at trace levels in soils. *P. vittata* is more efficient at As levels below 6 ppm in soils, and increased up to 40% higher than normal when As reaches up to 100 ppm (Wei and Chen, 2007).

In most studies, phytoremediation of contaminated sites has been evaluated using vascular plants, which in most cases may not be native plants. The use of non native plants requires adaptation procedures such as soil adjustment in order to survive the intent of planting, and for which studies revealed less than 60% plant survival (SERG, 2011). Moreover, phytoremediation with vascular plants may require soil amendments to effect clean up. Very little information is available on the use of forbs for phytoremediation procedures, perhaps because of their short life cycle. The short life cycle can be exploited for fast and efficient phyto-procedures if optimized and modified to cash in on a number of cycles for sequential remediation of contaminated sites (SRCSs), once the effective and best performing forbs are identified.

In this study, the potential of *P. maximum* (Guinea grass), a native and non vascular plant common in rain forest edge habitat to savannah grass land was evaluated for phytoaccumulation of Pb, Cr and Cd on moderately fertile contaminated soil.

MATERIALS AND METHODS

Soil collection and the determination of its physico-chemical properties

Soils were collected from the College of Animal Science and Livestock Production farm (less fertile soil) and Fadama farm (fertile soil) at the University of Agriculture Abeokuta, Ogun State, Southwest Nigeria. The soils were air dried, thoroughly mixed, and then subjected to physical-chemical analysis to determine the pH (McLean, 1982), conductivity (IITA, 1979), particle size distribution and mixed soil textural classification (IITA, 1979), exchangeable cation exchange capacity (ECEC) (Stewart, 1989), organic matter (Walkey and Black, 1934), total nitrogen and available phosphorus (Zhao et al., 1994).

Sources and cultivation of *Panicum maximum*

Five kilograms each of the blended soils were introduced into 72 units of 7 L capacity experimental pots which were segregated into six groups of 12 pots each. Each group of 12 pots was further subdivided in a 3x4 matrix (three groups of four pots). To each pot, about one week old juvenile guinea grasses (collected from nearby bush) were quickly transplanted (three stalks per pot) and the grasses nurtured to survival, and growth stabilization for one week.

Determination of phytoremediation potential of *Panicum maximum*

Five of the six groups of segregated experimental pots were exposed to different concentrations of Cd, Pb and Cr (20, 50, 75, 100 and 120 ppm, respectively) with daily wetting. The sixth group had no treatment and was used as experimental control. After the heavy metal treatment, plants were harvested from each group on a weekly interval and the various parts (roots, stem and foliages). The concentrations of Cd, Pd and Cr in roots, stems and foliage tissues of the harvested plants were determined according to the methods of Zhao et al. (1994). The soil resident concentrations of the heavy metals were measured weekly according to the method of Onianwa (2000).

Determination of metals bioaccumulation factors (BAF)

The bioaccumulation factor (BAF) defining the transport of metals from soil to different parts of *P. maximum* tissue were determined by the ratio of the metal concentration in plant tissue part to that in soil (BAF = $[metal]_{plant\ tissue}/[metal]_{soil}$) for each experimental pot.

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Table 1. Result of chemical analysis on soil.

Parameter	Minimun value	Maximum value	*Ibadan, Nigeria	**Southeast Ethiopia
pH	6.30	6.50	7.24 - 7.26	6.3 - 7.1
Electrical Conductivity (µS/cm)	94	105	-	-
Exchangeable Ca ($cmolkg^{-1}$)	1.07	1.38	3.28 - 3.44	2.41 - 3.50
Exchangeable Mg ($cmolkg^{-1}$)	0.85	1.10	1.20 - 1.27	0.65 - 0.73
Exchangeable K ($cmolkg^{-1}$)	0.12	0.18	1.28 - 1.43	0.30 - 0.41
Exchangeable Na ($cmolkg^{-1}$)	0.10	0.12	1.66 - 2.05	0.14 - 0.21
Organic carbon (%)	1.90	2.00	0.96 – 4.95	0.76 - 1.70
Available phosphorus (P) (µg/g)	5.82	7.50	11.97 - 12.59	-
Total nitrogen (N) (g/kg)	0.95	1.20	0.20 - 0.39	0.10 – 0.12
Soil class	Sandy loam	Sandy loam	Sandy loam	Sandy clay – clay soil

Data from *Eludoyin and Wokocha, 2011; **Belachew and Abera, 2010.

RESULTS AND DISCUSSION

Physico - chemical properties of soils

Physical examination conducted on the soil showed that the soil is porous and well aerated. This is as a result of the soil mixing process, which distorted the surface compaction order at soil collection, thus losing its confined nature; with concomitant effect on some geophysical and engineering properties of the soil. The soils are typically sandy loam based on particle size distribution analysis with 75.5, 17.0 and 7.5% sand, silt and clay, respectively.

Analysis results (Table 1) showed that the soils are slightly acidic with a pH range of 6.30 to 6.50. According to Romkens and De Vries (1995), this pH may allow for fair mobility of metals, though metal mobility and bioavailability also depends on their chemical form in soils (Ma and Rao 1997), and the soil physical properties. The electrical conductivity of the soil is moderate (94 to 105 µS/cm). The level of organic carbon (OC) (1.90 to 2.00%); available phosphorus (P) (5.82 to 7.50 µg/kg) and total nitrogen (N) (0.95 to 1.20 µg/kg) of the soil were moderate when compared to the findings of Eludoyin and Wokocha (2011) and Belachew and Abera (2010) (Table 1). The soil concentrations of the exchangeable cations or basic metals (Na, K, Ca and Mg) are within the normal range available in arable agricultural soils (Brady and Weil 2002).

Phytoremediation potential of *Panicum maximum*

The summary of the concentrations of Pb, Cr and Cd detected in the tissues: root, stem and foliage of *P. maximum* and soils are presented in Table 2. The uptake of Pb, Cr and Cd increased steadily in the root, stem and foliage system of *P. maximum* planted on the soil exposed to different level of metal treatment.

Results showed that there was a general decrease in soil concentrations of Pb, Cr and Cd, with attendant increase in the metal concentrations in different part of *P. maximum* over the 28 day study period. The roots of *P. maximum* retained the highest concentration of all metals investigated, with tissue abundance decreasing in the order Cr > Pb > Cd. The concentrations (µg/g) of Pb, Cr and Cd in soils; roots; stems; and foliages of the harvested *P. maximum* in experimental control (that is, plants in soils not spiked with the metals) sampled on the seventh, fourteenth, twenty-first and twenty-eight day were [0.28, 0.30, 0.24]; [0.08, 0.11, 0.06]; [0.06, 0.08, 0.04] and [0.02, 0.05, 0.01], respectively. Hattab et al. (2013) reported that the predication of the concentration of Cr in soil depends on some soil factors such as pH, EC, DOC etc. and the type of treatment or amendments to which the soil is subjected.

Over the four-week period of sampling, the levels of Pb decreased from 1.40 to 1.05 µg/g and from 1.57 to 1.30 µg/g, in soils, treated with 20 and 120 ppm of Pb, respectively. However, uptake of the Pb by *P. maximum* increased from 0.21 to 0.38 µg/g in the root, 0.18 to 0.30 µg/g in the stem and 0.09 to 0.18 µg/g in the foliage. The metal bioaccumulation factors (BAF) were 0.21 to 0.45 in root, 0.17 to 0.35 in stem and 0.08 to 0.21 in foliages of plants grown on soil treated with 20 ppm Pb, compared with soil levels of Pb measured weekly. Thus *P. maximum* accumulated between 13 to 45% of the soil concentration level. However, the levels detected in different plant tissues increased as soil treatment concentration of Pb increased. For example, soils treated with 120 ppm Pb showed increase in concentration level which ranged between 0.64 to 0.74 µg/g in the roots, 0.53 to 0.63 µg/g in the stem and 0.21 to 0.34 µg/g in the foliages, with corresponding BAF of 0.50 to 2.26 in the roots, 0.41 to 1.94 in stem and 0.03 to 1.00 in the foliages, compared to soil levels of Pb measured weekly.

The concentration of Cr in the treated soils diminished from 1.01 to 0.85 µg/g and 1.28 to 1.20 µg/g in soils

Table 2. Concentration levels (µg/g) of lead, chromium and cadmium in soil and different tissue part of guinea experimental grass .

Parameter	Control			Group 1 20 ppm Treatment			Group 2 50 ppm Treatment			Group 3 75 ppm Treatment			Group 4 100 ppm Treatment			Group 5 120 ppm Treatment		
	Pb (µg/g)	Cr (µg/g)	Cd (µg/g)	Pb (µg/g)	Cr (µg/g)	Cd (µg/g)	Pb (µg/g)	Cr (µg/g)	Cd (µg/g)	Pb (µg/g)	Cr (µg/g)	Cd (µg/g)	Pb (µg/g)	Cr (µg/g)	Cd (µg/g)	Pb (µg/g)	Cr (µg/g)	Cd (µg/g)
Week 1																		
Soil	1.38	0.35	0.28	1.40	1.01	0.90	1.44	1.08	0.92	1.48	1.14	0.94	1.52	1.22	0.97	1.57	1.28	1.00
Root	0.08	0.11	0.06	0.21	0.43	0.13	0.34	0.56	0.17	0.47	0.60	0.21	0.52	0.71	0.22	0.64	0.82	0.25
Stem	0.06	0.08	0.04	0.18	0.24	0.09	0.25	0.31	0.12	0.35	0.42	0.15	0.41	0.56	0.18	0.53	0.63	0.21
Leaf	0.02	0.05	0.01	0.09	0.11	0.05	0.11	0.15	0.07	0.14	0.17	0.09	0.17	0.20	0.11	0.21	0.29	0.15
Week 2																		
Soil	1.36	0.33	0.26	1.12	0.97	0.67	1.15	1.00	0.71	1.20	1.10	0.74	1.30	1.18	0.80	1.33	1.25	0.83
Root	0.10	0.14	0.08	0.28	0.35	0.15	0.30	0.44	0.17	0.43	0.48	0.22	0.56	0.61	0.25	0.10	0.14	0.08
Stem	0.08	0.12	0.05	0.22	0.27	0.10	0.26	0.32	0.12	0.35	0.37	0.15	0.44	0.49	0.21	0.08	0.12	0.05
Leaf	0.04	0.06	0.03	0.13	0.15	0.06	0.15	0.17	0.08	0.18	0.19	0.10	0.20	0.24	0.14	0.04	0.06	0.03
Week 3																		
Soil	0.34	0.31	0.26	1.08	0.95	0.65	1.11	0.98	0.68	1.16	1.07	0.71	1.27	1.16	0.77	0.34	0.31	0.26
Root	0.13	0.15	0.09	0.31	0.38	0.18	0.32	0.41	0.22	0.45	0.54	0.25	0.60	0.63	0.27	0.70	0.66	0.28
Stem	0.11	0.12	0.07	0.27	0.31	0.11	0.29	0.35	0.20	0.38	0.42	0.16	0.48	0.46	0.18	0.60	0.48	0.21
Leaf	0.06	0.37	0.06	0.15	0.20	0.08	0.18	0.24	0.10	0.21	0.26	0.12	0.24	0.28	0.14	0.31	0.35	0.17
Week 4																		
Soil	0.38	0.30	0.24	1.03	0.85	0.62	1.10	0.92	0.73	1.12	1.02	0.75	1.22	1.13	0.79	1.30	1.20	0.81
Root	0.14	0.17	0.12	0.38	0.71	0.33	0.41	0.73	0.35	0.51	0.77	0.37	0.63	0.80	0.41	0.74	0.83	0.43
Stem	0.12	0.14	0.11	0.30	0.53	0.27	0.32	0.56	0.28	0.42	0.59	0.30	0.50	0.62	0.33	0.63	0.64	0.35
Leaf	0.07	0.01	0.09	0.18	0.35	0.23	0.21	0.39	0.24	0.25	0.42	0.27	0.27	0.45	0.28	0.34	0.47	0.31

treated with 20 and 120 ppm of Cr, respectively over the four week study period. The uptake of Cr by *P. maximum* increased from 0.43-0.71 µg/g in root, 0.24-0.53 µg/g in stem and 0.11 to 0.35 µg/g in foliages of plant in soils treated with 20 ppm. The BAF of Cr in different part of *P. maximum* also increased over the study duration and ranged between 0.31 to 0.69 in the root, 0.17 to 0.52 in the stem and 0.07 to 0.34 in the foliages of plant sample grown on 20 ppm Cr - treated soils. This showed that *P. maximum* accumulated between 13 to 69% of the heavy metals. The detected concentration levels of Cr in the different plant tissues increased as soil treatment concentration of Cr increased. The increase in Cr concentration observed in tissues of *P. maximum* in soils treated with 120 ppm Cr which ranged: 0.14 to 0.83 µg/g in root, 0.24 to 0.53 µg/g in stem and 0.63 to 0.64 µg/g in foliages, with corresponding BAF of 0.11 to 1.94 in root, 0.09 to 0.49 in stem and 0.05 to 1.03 in foliage compared with soil levels of Cr measured weekly.

The concentration levels of Cd increased in tissues of *P. maximum* over the study period,

while Cd levels in soils decreased. Soil levels of Cd dropped from 0.90 to 0.24 µg/g and from 1.00 to 0.81 µg/g in soils treated with 20 and 120 ppm Cd respectively. The uptake of Cd by *P. maximum* increased from 0.13 to 0.33 µg/g in the roots, 0.09 to 0.27 µg/g and 0.05 to 0.23 µg/g in the foliages. Cadmium BAF in *P. maximum* were 0.14 to 0.53 in the roots, 0.10 to 0.44 in stem and 0.05 to 0.37 in foliages of plant grown in soils treated with 20 ppm Cd compared with soil levels of Cd measured weekly. Cadmium accumulation in *P. maximum* however lies between 9 to 53%. However the levels observed in different plant tissues increased as soil treatment concentration of Cd increased. For instance, soils treated with 120 ppm Cd showed increase in concentration which ranged between 0.25 - 0.43 µg/g in the roots, 0.21 to 0.35 µg/g in the stem and 0.15 to 0.31 µg/g in the foliages, with corresponding BAF of 0.25 to 1.08, 0.21 to 0.82 and 0.04 to 0.65, respectively, compared to soil levels of Cd measured weekly.

The results showed that the rate of metals uptake or absorption by the plant is proportional to the metal concentration in the soils and plant age i.e. duration of exposure.

Performance of *P. maximum* on metal contamination soil

The growth profiles of *P. maximum* were steady with an average rate of height increase of 20 cm per week, and reaching a maximum height range of between 100 and 120 cm over the four-week period. The plants were green and appeared unaffected, except group 5 plants whose foliages experienced leave burning after the third week resulting into yellowing-browning of the foliages. Consequently, the growth pattern of *P. maximum* plants used in this study were considered to be above average, as their growth rate were observed to be consistent with the report of FAO (2003) and Humphrey and Patridge (1995) who noted rapid growth reaching a height of about 1.5m during the first four weeks.

Profile of Pb, Cd and Cr in different plant tissues

The concentrations of Pb, Cr and Cd detected in the different plant parts (root, stem and foliage) increased over the study period, except for Pb and Cd which showed a sudden down surge of concentrations in roots, stems and foliage at week 2 harvest in group 5 plants (120 ppm Pb and Cd treated soils), compared to the detected levels in week 1 harvest. Higher concentrations were measured in roots, stems and foliages of *P. maximum* planted on most of the contaminated soils reaching 100 ppm Pb, Cr and Cd. Thus, the uptake pattern of Pb, Cr and Cd by *P. maximum* appeared to be a function of their concentrations in their respective soil environment. This is consistent with the findings of Page et al. (1981) and Kabata-Pendias et al. (1993), which implies that metals uptake by *P. maximum* is dependent on the degree of metal contamination in soil. On the contrary, soil concentration of Pb, Cr and Cd decreased over the study period. Also the concentration levels of Pb, Cr and Cd available in *P. maximum* decrease in the order root > stem > foliage. The concentrations of Pb, Cr and Cd also increased steadily in the different parts of *P. maximum* with duration (plant experimental age) from day seven today twenty eight during the harvest periods. The highest concentrations were noted in soils spiked each with 120 ppm of Pb, Cr and Cd (Experiment group 5 pots) compared with the other treatment groups.

Expression of metal toxicity in the form of foliage damage or burns (resulting in yellowish-brown foliage) appeared in *P. maximum* planted on soil treated with 120 ppm Pb and Cd (experiment group 5), on day 23 (about week 3). Concentration of Cd and Pb reaching 120 ppm appeared to be phytotoxic to the plant. The phytotoxic point is the concentration level at which the plant begin to manifest obvious toxicity effect from the contaminant. Apparently, there was obvious cytotoxic damage in *P. maximum* leading to loss of control in metal uptake at this treatment concentration. This occurred at some point after day fourteen, leading to excessive levels of Pb in tissues of *P. maximum* which showed up to 226% Pb compared with soil level at day twenty-one. This signifies the maximum holding capacity of *P. maximum* at 56% soil level, in 100 ppm treatment 5 kg soils if other conditions are optimized. This suggest that the concentrations of the toxic metals in soil is not as much an issue in the evaluation of phytoremediation potential of plant species, than the levels at which phytotoxic response of plant appears during remediation process. It is also not absolute to conclude that the foliage burns effect is a response to high concentration levels of Cd and Pb, because the experimental soils used. Metal uptake and accumulation by *P. maximum* also need to be tested on high nutrient quality soils at concentration of 120 ppm and above for of Cd and Pb removal.

Therefore, the potential for uptake of Pb, Cr and Cd by *P. maximum* when planted on heavy metals contaminated soils is high and when contamination is moderate, because high metal concentration may be phytotoxic to the plant at some point.

Conclusion

Phytoremediation of metal contaminated soils with *P. maximum* showed a promising potential under the conditions of experiment. The distribution and accumulation of metals in the plants were variable, with the root tissues accumulating significant concentration of the Cr, Pb and Cd than the stem and the foliar tissues. Obvious signs of phyto-toxic effect appeared in plants treated with 120 ppm Pb and Cd at day 23, which implies that *P. maximum*

may not be metal hyper accumulator. It is however important to note that the selection of promising plants is critical to success of phytoremediation. Further work is recommended to evaluate the effect of soil amendments on the response of *P. maximum* to metal uptake and plant performance. Positive outcome will further reduce technical and human input in phytoremediation procedure as well as reduce clean up duration since the cycle of *P. maximum* is perennial.

REFERENCES

Azevedo RA, Gratao PL, Prasad MNV, Cardoso PF, Lea PJ. (2005). Phytoremediation: green technology for the clean-up of toxic metals in the environment. Braz. J. Plant Physiol. 17(1): 53-64.

Belachew T, Abera Y (2010). Assessment of soil fertility status with depth in wheat growing highlands of Southeast Ethiopia. World J. Agric. Sci. 6(5): 525–531.

Brady N, Weil R (2002). The nature and properties of soils, 13th Edition. Prentice Hall, Upper Saddle River, New Jersey. 960 p.

Chen YH, Francis JA, Miller JR (2002). Surface temperature of the Arctic: Comparison of TOVS satellite retrievals with surface observations. J. Climate. 15: 3698–3708

Davies FT Jr, Puryear JD, Egilla JN, Saraiva Grossi JA (2002). Mycorrhizal fungi increase chromium uptake by sunflower plants: influence on tissue mineral concentration, growth and gas exchange. J. Plant Nutr. 25(11): 2389-2407

Eludoyin OS, Wokocha CC (2011). Soil dynamics under continous monocropping of maize (*Zea mays*) on a Forest Alfisol in South-Western Nigeria. Asian J. Agric. Sci. 3(2): 58–62. ISSN: 2041–3890

Environmental Protection Agency (EPA) (2000). Appendix 5: Phytoremediation [cited 2005, Feb 15] Available from http://www.psparchives.com/publications/our wo

Fayiaga AO, Ma LQ, Rao X, Rathinasabapathi B (2004). Effects of heavy metals on growth and arsenic accumulation in the arsenic hyperaccumulator *Pteris vittata L.* Environ. Pollut. 32(2): 289-296.

Food Agricultural Organisation (FAO) (2003). *Panicum maximum*, guinea grass, colonial grass, Tanganyika grass. [cited 2007, June 20] Available from http://www.fao.org/ag/aga/agap/frg/AFRIS/DATA/118.html.

Hattab N, Hambli R, Motelica-Heino M, Bourrat X, Mench M (2013). Application of neural network model for the prediction of chromium concentration in phytoremediated contaminated soils. J. Geochem. Expl. 128: 25-34.

Hattab N, Hambli R, Motelica-Heino M, Bourrat X, Mench M (2013). Neural network and Monte Carlo simulation approach to investigate variability of copper concentration in phytoremediated contaminated soils. J. Environ. Manag. 129(15): 134-142.

Hemen S (2011). Metal hyperaccumulation in plants: A review focusing on phytoremediation technology. J. Environ. Sci. Technol. 4: 118-138.(Year in reference does not correspond with that in the work).

Humphrey LR, Patridge IJ (1995). A Guide to better pastures for the tropics and sub tropics. Published by NSW Agriculture 5th edition: Grasses for the tropics: Guinea grass (*Panicum maximum*).

International Institute for Tropical Agriculture (IITA), (1979). Selected methods for soil and plant analysis, Manual series No 1, Nigeria.

Kabata-Pendias A, Piotrowska M, Dutka S (1993). Trace element in legumes and monocotyledons and their suitability for the assessment of soil contamination. In Markert B (ed), Plants as biomonitors. Indicators for heavy metals in the terrestrial environment Third edition. pp. 485-494.

Li MS (2005). Ecological restoration of mineland with particular reference to the metalliferous mine wasteland in China: A critical review of research and practice. Sci. Total Environ. 357(1 - 3): 38–53.

Li Y, Dhankher OP, Carreira L, Lee D, Chen A, Schroeder JL, Balish R, Meagher RB (2004). Over expression of phytochelatin synthase in aribidopsis leads to enhanced arsenic tolerance and cadmium hypersensitivity. Plant Cell Physiol. 45(12): 1787- 1797.

Lyubun YV, Fritzsche A, Chernyshova MP, Dudel EG, Fedorov EE (2002). Arsenic transformation by *Azospirillum Brasilense* Sp245 in Association with wheat (*Triticum Aestivum* L.) roots. *Plant and Soil* 286(1 - 2): 219–227.

Ma OL, Rao NG (1997). Chemical fractionation of cadmium, copper, nickel and zinc in contaminated soils. *J. Environ. Qual.,* 26: 259-264.

McLean EO (1982). Soil pH and lime requirement. In: Page AL, Ganje TJ, Joshi MS (Eds.). Methods of soil Analysis, Part 2, Agronomy 9, *American Society of Agronomy Inc.*, Madison, Winconsin, USA. 17: 199–224.

Onianwa PC (2000). Roadside topsoil concentration of lead and other heavy metals in Ibadan, Nigeria. Soil Sed. Contam. 10(6): 577-591

Page CG, Bennetts AJ, Ricketts MJ (1981). European Space Agency, ESLAB Symposium on X-ray Astronomy, 15th, Amsterdam, Netherlands. Space Sci. Rev. 30 (1-4): 369-371.

Romkens PF, De Vries W (1995). Acidification and metal mobilization: Effect of land use changes on cadmium mobility. Environ. Sci. 64:367-380.

Sizova OI, Kochetkov VV, Boronin AM (2006). The arsenic-phyto-remediation potential of genetically modified *pseudomonas spp.*: Phytoremediation of metal-contaminated soils: NATO Science Series: IV: Earth Environ. Sci. 68: 327-334.

Soil ecology and restoration group (SERG) (2000). 29 Palms Tank Trail Project. [cited 2011, Mar 25] Available from http://www.sci.sdsu.edu/SERG/restorationproj/mojave

Stewart EA (1989). Chemical analysis in ecological materials, 2nd ed., London Blackwell Scientific, Oxford.(Year in referene does not correspond with that in the work.)

Walkey A, Black IA (1934). An examination of the digestion method for the determination of organic matter and a proposed chromic oxide titration. Soil Sci. 37: 29–38

Wei CY, Chen TB (2007). Arsenic accumulation by two brake ferns growing on an arsenic mine and their potential in phytoremediation. Chemosphere. 63(6): 1048- 1053

Zhao F, McGrath SP, Crosland AR (1994). Comparison of three wet digestion methods for the determination of plant sulphur by inductively coupled plasma atomic emission spectrometry (ICP-AES). *Commun.* Soil Sci. Plant Anal. 25: 407-418.

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Application of mixed models for the assessment genotype and environment interactions in cotton (*Gossypium hirsutum*) cultivars in Mozambique

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In the process of introducing cotton cultivars, it is essential to assess their productive behavior for different environments for which they will be recommended. Knowledge of the magnitude of the genotype interaction with environment allows the evaluation of the stability and adaptability of genotypes where one intends to introduce them, in addition to enabling the evaluation of the production potential and possible limitations of each environment. The study was conducted to determine the productivity, genotypic adaptability and genotypic stability of nine cotton cultivars *(Gossypium hirsutum*) in Mozambique, from 2004 to 2010 growing seasons. The genotypic stability and genotypic adaptability were assessed by Residual Maximum Likelihood (REML) and predict breeding values using Best Linear Unbiased Prediction (BLUP) methodology. The cultivars ISA 205, STAM 42 and REMU 40 showed superior productivity when they were selected by the Harmonic Mean of Genotypic Values (HMGV) criterion in relation to others. In turn, the cultivars CA 222, STAM 42 and ISA-205 were superior when selected by the Relative Performance of Genotypic Values (RPGV) and Harmonic Mean of the Relative Performance of Genotypic Values (HMRPGV). The cultivars CA 324 had the lower values for all criterions above. The cultivars CA 222 and STAM 42 will be the most recommended for farmers in cotton-growing regions and for the Cotton Breeding Program of Mozambique.

Key words: *Gossypium hirsutum*, harmonic mean of the relative performance of genotypic values (HMRPGV), relative performance of genotypic values (RPGV), harmonic mean of genotypic values (HMGV), residual maximum likelihood (REML)/best linear unbiased prediction (BLUP).

INTRODUCTION

Cotton (*Gossypium hirsutum)* is currently the leading crop in natural fiber production and is grown commercially in several environments, both in temperate as well as in tropical climate areas (Park et al., 2005; Naveed et al.,

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2007; Khadi et al., 2010). Cotton is the fifth crop for oil production, and the second for protein source in the world (Wallace et al., 2008) and the fiber´s ginning of 1.0 kg can be obtained by 1.65 kg of seed contain 21% oil and 23% protein (Benbouza et al., 2010). There are about 60 countries around the world that cultivate cotton in 34 million hectares. The countries include Australia (2, 000 kg ha^{-1}), Brazil (1, 338 kg ha^{-1}), China (1, 265 kg ha^{-1}), Mexico (1, 247 $kgha^{-1}$), United States of America (985 kg ha^{-1}), Uzbekistan (831 kg ha^{-1}), Pakistan (599 kg ha^{-1}) and India with 550 (kg ha^{-1}) (Fengguo et al., 2007; Khadi et al., 2010). The genus *Gossypium* includes approximately 50 species distributed worldwide, in the following continents: Asia, Africa, Australia and America, from which five are tetraploid species and belongs the subgenus viz. *Karpas* (Brubaker and Wendel, 1994; Cronn and Wendel, 2004). Among these species, only four are exploited economically: G. *herbaceum*, G. *arboretum*, G. *barbadense* and G. *hirsutum*, the latter contributing around 90% of the world output of cotton (Zhang, 2008).

The Mozambique Cotton Breeding Programs have focused mainly on the yield of cottonseed and fiber, with the CA 324 and REMU 40 cultivars are widely used by farmers, which together representing about 80% of the total cotton growing area (Bias and Donovan, 2003; IAM, 2009; Maleia et al., 2010). Although some of these introduced cultivars are already being used by producers because of incentive from fomenting companies, they only have been assessed by phenotypic stability and adaptability with balanced data (Maleia et al., 2010). In this sense, the methodology of mixed models, which allows the use of unbalanced data, and is widely used in breeding programs of perennial plants, becomes very important tools or methods to evaluate a performance of annual plants (Mora et al., 2007; Piepho et al., 2008), as cotton.

The use of mixed linear models in the advanced stages of cultivar selection such as in cultivation and use value of genotypes, which are set up in various environments, has fundamental importance, furthermore, the use of BLUP is preferable to the *Best Linear Unbiased Estimator* (BLUE) (Piepho and Möhring, 2006). In current studies, the genetic effect has been referred as random (Resende, 2007), allowing therefore the estimation of variance components, obtainment of genotypic values and the use of linear mixed linear models (Piepho et al., 2008).

The interaction of genotype and environment interferes significantly in breeding programs (Cruz and Carneiro, 2006) as an ideal cultivar, should be adapted to a broad cultivation environment (Cruz, 2005; Cruz and Carneiro, 2006). However, the interaction, in most cases, allows the release of cultivars for specific environments where they have a greater adaptation (Campbell and Jones, 2005). Therefore, knowledge of the magnitude of interaction genotype with environment is important to assess the stability and the adaptability of genotypes where they are intended to be introduced (Contreras and Krarup, 2000) also allows to evaluate the production potential, and possible limitations of these in each environment (Mora et al., 2007).

The simultaneous evaluation of stability and adaptability in the context of Mixed Linear Models (Resende, 2007) can be carried out using the HMRPGV Predicted (Silva et al., 2012). Although, the use of the REM/BLUP Methodology, the HMRPGV Method can be used for analysis of unbalanced data (Resende, 2007), non-orthogonal designs (Piepho et al., 2008), and designs with heterogeneity of variance (Mendes et al., 2012). This type of evaluation for commercial cotton cultivars is scarce in Mozambique. Therefore, the objective of this study was to evaluate the interaction between genotypes and environments, productivity, genotypic adaptability and genotypic stability of cotton cultivars in Mozambique, using the Mixed Models (REML/BLUP).

MATERIALS AND METHODS

Location of the experiments and sowing dates

The experiments were set up in the municipality of Montepuez, in the Namialo and Namapa Villages, located in the Northern Region of Mozambique, from growing season 2003/2004 to 2009/2010. In Morrumbala village, in the central region of the country, the experiments were set up from growing season 2005/06 to 2009/2010. All the locations are situated in Agro-ecological Regions 6, 7 and 8 (INIA, 2000).

The Agro-ecological Region 6 (R6) represents the semi-arid region of the Zambezi Valley and Southern of Tete Province Mozambique, which is a vast dry area. In contrast, the Agro-ecological Region 7 (R7) is region of medium-altitude in Zambezia, Nampula, Tete, Niassa and Cabo-Delgado Province Mozambique, with a variable soil texture. There is great potential for cotton production which has been practiced for several decades. The Agro-ecological Region 8 (R8) represents the Coast of Zambezia, Nampula and Cabo Delgado Provinces Mozambique, and the soils are generally sandy in some areas. The low soil fertility is one of the great limiting factors in these areas (INIA, 2000).

The municipality of Montepuez is located in the Agro-ecological Region R7, at an altitude of 555 m (medium altitude), 38º59' Longitude East and 13° 07' Latitude South, in the District of Montepuez, in the Southern of the Cabo Delgado Province Mozambique. Namialo is located between the Agro-ecological Regions R7 (medium altitude) and R8 (Coastal side) at an altitude of 157 m, 39° 59' Longitude East and 14° 55' Latitude South, in the Meconta District, Central Eastern of Nampula Province, Mozambique.

The Namapa Village is located in the Eráti District, at an altitude of 200 m (Low altitude), 13° 43'S Latitude and 39° 50' Longitude East between the R7 and R8 Agro-ecological Regions in the North of Nampula Province, Mozambique. The Morrumbala Village, in turn, is located between the Agro-ecological Regions R6 (semi-arid of Zambezi Valley) and R7 (medium altitude), at an altitude of 392 m, 35° 35' Longitude East and 17° 19' Latitude South, in the Morrumbala Village, in the Lower Region of Zambezia, Zambezia Province, Mozambique.

Climate and soil

The Namialo region is characterized by an Aw climate type

Table 1. List of cultivars assessed, origin, year of introduction, tolerance characteristics to *E. fascialis*, lint outturn, growing season.

Cultivar	Origin	Year of introduction	Tolerance to *Empoasca fascialis*	Lint outturn-GOT (%)	Growing season (days)
ALBAR SZ9314	Zimbabwe	1999	High	>42	>150
ALBAR FQ902	Zimbabwe	1999	High	41	130-150
ALBAR BC853	Zimbabwe	1999	High	37	<130
STAM 42	Senegal	1999	Low	40	130-150
CA 222	Ivory Coast	1994	Medium	39	130-150
CA 324	Ivory Coast	1994	Medium	38	130-150
IRMA 12-43	Cameron	1994	High	39	130-150
ISA 205	Ivory Coast	1994	High	39	130-150
REMU 40	Mozambique	1980	High	37	130-150

Source: IAM, 2007; Maleia et al., 2010.

(Köppen, 1948), dry sub-humid where the annual rainfall ranges from 800 to 1,000 mm and the average annual temperature of about 26°C. The soils classification ranges from sandy (ferralic arenosols and sandy textured haplic arenosols) to sandy clay and gleyic arenosols that occur alternately with hydromorphic sandy soils (MAE, 2005a). The Montepuez region has an Aw climate type (Köppen, 1948), semi-arid to sub-humid, with average annual precipitation ranging from 800 to 1,200 mm and the mean annual temperature ranging from 20 to 25°C. The hydromorphic soils predominate in this region, whose texture ranges from sandy, sandy on clay, and molic type dark-colored stratified soils gleic and dristic to halpic and luvic phaeosems (MAE, 2005b).

The Morrumbala region has an Aw climate type (Köppen, 1948), rainy tropical savanna with mean annual temperature of 22°C and 1,000 mm of rainfall. Soils are predominantly red, ranging from lightly sandy to clay, with deep ferralic lithosols (MAE, 2005c). The Namapa region has an Aw climate type (Köppen, 1948), semi-arid to sub-humid, with average annual rainfall that may exceed 1,500 mm and the mean annual temperature ranging from 20 to 25°C. The hydromorphic soils predominate, whose texture ranges from dark to gray sandy, sandy clay and stratified clay (MAE, 2005d).

Experimental design

To implement these experiments in the 19 study environments, a randomized complete blocks design was used. Each of three or four replicates (unbalanced data) consisted of set nine commercial cultivars. Table 1 shows nine cultivars and the mainly agronomic characteristics. In all experiments, plant to plant space was 0.2 m and row to row space was 1.0 m which corresponded to 50 000 plants ha^{-1} population density (Carvalho, 1996; IAM, 2007). The useful area of each plot consisted of three central rows, covering a usable area of 15 m². The experiments were set up in a non-irrigated area during the beginning of the rainy season, usually in the first two weeks of December.

Planting and other agronomic practices

The sowing was carried out manually in the hill plot (using a hoe) in rows, placing four to ten seeds per hill plot, approximately 4 cm deep. The first thinning was at fifteen days after seedling emergence, leaving two plants per hill plot. Later, at 21 days after emergence, a second thinning was performed leaving only one plant per hill plot. Weeds were controlled manually by hoeing five to six times, in order to prevent them from competing with the crop. No side dressing, mulching or fertilization was applied in order to allow experiments to simulate conditions similar to those prevailing in the rural producing fields in regions of Mozambique (Bias and Donovan, 2003). Two sprays with Endosulfan insecticide (475 $g.L^{-1}$), followed by three applications of Lambda-cihalothrin (50 $g.L^{-1}$) once in two weeks were applied (IAM, 2007), starting in the sixth week after emergence. The insecticides were applied using an ultra low volume nozzle (ULV).

Data collection

The characteristic evaluated was the total production of cottonseed harvested from all plants in the useful area of each experimental plot, with the mean value expressed in $Kg.ha^{-1}$.

Statistical analyses

The experimental data were test for normality and homogeneity of the errors (Levene, 1960; Shapiro and Wilk, 1965) in each environment using the SAS 9.2 software (SAS, 2009). The adaptability and stability were analyzed by the REML/BLUP Methodology (Henderson, 1975), considering the following statistical model:

$$E\begin{bmatrix} y \\ g \\ gl \\ \varepsilon \end{bmatrix} = \begin{bmatrix} Xr \\ 0 \\ 0 \\ 0 \end{bmatrix}; \quad \mathbf{var}\begin{bmatrix} \mathbf{g} \\ \mathbf{gl} \\ \boldsymbol{\varepsilon} \end{bmatrix} = \begin{bmatrix} \mathbf{I}\sigma_g^2 & 0 & 0 \\ 0 & I\sigma_{gl}^2 & 0 \\ 0 & 0 & I\sigma_{\varepsilon}^2 \end{bmatrix}$$

Where; Y is the vector of observation, *r* is the vector of fixed effects (replication) added to the overall mean and include all the repetitions of all places, g is the vector of random effects (genotypes), *gl* is the vector of effects of genotype x environment interaction (random), and $\mathcal{E}$ is the error vector (random). The X, Z and W, are associated design matrices for r, g, and gl, respectively.

The predicted genotypic values for genotype i at each site j simultaneously uses data from all environments, are given by $GV_{ij}=U_j+g_i+gl_{ij}$ where Uj is the average of location j. In this case, both g and gl are predicted because every data set is used, and the additional residues of interactions are eliminated when producing the Blup's of gl as well (Resende, 2007). The random effects are assumed to be distributed as:

$\mu \sim MVN(0; G)$ and $\varepsilon \sim MVN(0; R)$

Where, MVN (μ ; V) means multivariate normal distribution with mean μ and variance-covariance matrix V (Piepho et al., 2008).

In the simultaneous evaluation of genetic stability and adaptability of cotton cultivars was used the Harmonic Mean of the Relative Performance of the Genotypic Value (HMRPGV), as described by Resende (2007). These method is advantageous over methods such as Lin and Binns (1988) and Annicchiarico (1992), once it provides results that can be directly interpreted as genotypic values (Oliveira et al., 2005; Mora et al., 2007; Resende, 2007), allows to compute the composite character of genetic gain in the productivity, stability and adaptability (Resende, 2007). And also it does not depend on assumptions of α values associated with $Z_{(1-\alpha)}$, which refers to the percentile of the standard normal distribution function associated with a level of α, respectively (Rezende, 2007). The analysis of stability and adaptability were carried out using the software Selegen REML/BLUP (Resende, 2002).

Regarding the deviance analysis and estimation of the effect of genotypic and genotype x environment interaction, the PROC MIXED was applied (Littell et al., 2006). The estimator used for the prediction of genotypic values was the BLUP, which estimates variance's components of random factors obtained by the Method of Restricted Maximum Likelihood *(*REML) (Resende, 2007).

RESULTS AND DISCUSSION

The errors showed a normal distribution for each environment, but the variance analysis was not for overall environments allowing that the all analyzes were consider heterogeneous variances (Resende, 2007).

The Likelihood Ratio Test of the *Joint Analysis of Deviance* (Littell et al., 2006) for the productivity of cottonseed (Table 2) showed the effect of the cultivars as significant, and the coefficient of variation (CV) was 22.67%. This value of the coefficient of variation shows good precision of the experiment (Bowman, 2001), as the character cotton productivity is strongly influenced by the environment. In spite of, Maleia et al. (2010) when evaluating the adaptability and stability of the same cultivars used in this study and in 7 environments had a coefficient of variation of 18.39%. It is important to emphasize that for yield of cottonseed various authors estimate a coefficient with a range of 4.7 to 31.5%, and an average of 14.3% (Mora et al., 2007).

The genotype x environments interaction was significant (Table 2), indicating that cultivars showed different responses when exposed to different environments (local and production year) suggesting that the performance ranking of the cultivars was not constant. Table 3 shows the cultivars with their genotypic values for Namialo 2003/2004 and Namialo 2004/2005, and the overall environments analysis. It can be verified that the cultivar ALBAR BC853 showed the lowest genotypic value mean, that is, it had the worst performance to the overall environment. Considering the genotypic values (Table 3), the best cultivar in the different environments was the CA 222 cultivar. This ranking differs from that demonstrated by Maleia et al. (2010) when same genotypes were assumed as fixed effects in seven of the 19 environments assessed in this study, which concluded that the ISA 205 cultivar had the best productivity.

Table 2. Values of the Statistical Likelihood Ratio Test of the Joint Deviance Analysis and coefficient of experimental variation (CV (%) for cottonseed yield (kg ha^{-1}) in 19 environments, from 2003/2004 to 2009/2010 growing season.

Source of variation	F value
Cultivars	2.37 *
Environment x cultivars	3.98**
χ^2 Test	37.76**
CV(%)	22.67

The ISA 205 cultivar was highlighted in Namialo in 2003/2004 growing season (Table 3), while the STAM 42 cultivar showed a higher genotypic value in Namialo during 2004/2005 growing season, thus demonstrating the presence of genotype x environment interaction. Although, Maleia et al. (2010) referred that cultivar ISA 205 had the major value in the Namialo during 2003/2004 growing season, while in the Namialo environment during 2004/2005 growing season, were the cultivar STAM 42, assuming the genotypes effects as fixed.

This similarity is regarding to the normal distribution of the errors and the homogeneity of variance, consequently, the ranking of the cultivars obtained by the REML/BLUP methodology were the same with the classical methodology (Oliveira et al., 2005; Mora et al., 2007; Rezende, 2007; Piepho et al., 2008). Furthermore, Piepho and Möhring (2006) demonstrated that the use of BLUP is preferable to the BLUE.

Table 4 shows the results penalizing or capitalizing cultivars according to their performance in relation to stability (HMGV) for the overall environments. With respect to stability, it has been found that cultivars ISA 205, STAM 42 and REMU 40 had a superior HMGV, whereas the cultivars with smaller genotypic values for stability (HMGV) were cultivars CA 324, ALBAR BC853. The Maleia et al. (2010) showed a different ranking of cultivars from what obtained in present study, since ISA 205, STAM 42 and IRMA12-43 cultivars had value above 100% when estimated by the W_i confidence index, evidencing a greater phenotypic stability for cottonseed yield. In this study, the productive superiority for genotypic stability belongs to ISA 205, STAM 42 and REMU 40 cultivars. In relation to the REMU 40 cultivar, which is originated from Mozambique and widely produced (Bias and Donovan, 2003; IAM, 2009). Its superiority was obtained when selected by the HMGV method, however, Maleia et al. (2010) when using the Annichiarico (1992) method did not point out this superiority. The same authors recommended the STAM 42 cultivar for low quality environments, as it showed phenotypic adaptability restricted to those environments. In contrast, the STAM 42 cultivar showed superiority for both productive adaptability and stability in this study.

Table 3. Genotypic values obtained by the REML/BLUP methodology of cottonseed productivity (kg. ha^{-1}) in 19 environments, from 2003/2004 to 2009/2010 growing season, regarding the (u+g) predicted genotypic values, free of interaction with environments, and genotypic values predicted by environment (u+g+ge).

Cultivar	Overall environment		Namialo 2003/2005		Namialo 2004/2006	
	u + g	New mean	u+g+ge	New mean	u+g+ge	New mean
ALBAR SZ9314	1,530.52	1,542.76	837.11	871.03	1,336.53	1,411.59
ALBAR FQ902	1,521.13	1,535.43	839.58	877.82	1,439.31	1,447.51
ALBAR BC853	1,474.90	1,528.71	796.91	857.39	1,038.03	1,337.03
STAM 42	1,543.25	1,549.02	866.84	896.61	1,459.10	1,459.10
CA 222	1,554.78	1,554.78	776.10	848.36	1,256.36	1,374.40
CA 324	1,526.72	1,540.09	836.04	866.03	1,269.34	1,391.27
IRMA 12-43	1,521.79	1,537.47	859.65	887.37	1,353.11	1,426.60
ISA 205	1,542.19	1,545.82	947.51	947.51	1,444.11	1,451.61
REMU 40	1,543.06	1,547.03	875.50	911.50	1,437.35	1,444.97

Table 4. *Stability of Genotypic Values* (HMGV) for cotton cultivars evaluated in 19 environments, between 2003/2004 and 2009/2010 growing season.

Cultivar	Genotypic value (HMGV)
ISA 205	1,447.74
STAM 42	1,445.97
REMU 40	1,437.65
CA-222	1,436.61
IRMA 12-43	1,413.04
ALBAR FQ902	1,399.81
ALBAR SZ9314	1,390.68
CA 324	1,389.33
ALBAR BC853	1,312.23

It is worth emphasizing that the CA 324 cultivar in this study showed inferior productivity for both genotypic stability and adaptability (Table 5). However, it has been recommended for the quality's environments by Maleia et al. (2010). The CA 222 cultivar was not referenced in the recommendations of the Maleia et al. (2010) evaluated in seven of 19 environments assessed in this study, when evaluating the adaptability and phenotypic stability. Such facts reveal that the RPGV and HMRPGV*GM (Global Mean) are more efficient than the methods as Lin Binns (1988) and Annichiarico (1992) in the evaluation of adaptability and stability, respectively.

Table 5 shows the results penalizing or capitalizing cultivars according to their performance in relation to adaptability, stability and adaptability jointly to overall environments. Cultivars CA 222, STAM 42 and ISA 205 presented higher values when selected by the RPGV and RPGV*GM Criterion (Resende, 2002), as well as for the HMRPGV and HMRPGV*GM Method (Resende, 2002), while the cultivars with lowest values were CA 324 and ALBAR BC853 for these method. The Cultivar CA 222, STAM 42 and ISA 205 should response, in general 1.02 times above in relation to the mean of the averages of the environments where they are grown, both for RPGV and HMRPGV (Table 5).

The CA 222, STAM 42 and ISA 205 cultivars may be the most suitable and most promising for farmers in Agro-ecological regions of Mozambique and for the cotton Breeding Program of Mozambique.

Conclusions

The REML/BLUP methodology enabled to determine the genotypic stability and genotypic adaptability of the nine cultivars even with unbalanced data and heterogeneity of variances of the errors. The genotypic values were higher in overall environments for CA 222 and STAM 42 cultivars.

The cultivars ISA 205, STAM 42 and REMU 40 showed the highest values of the cottonseed yield when selected by the HMGV method, while the lowest values for the CA 324 and ALBAR BC853 cultivars. In relation to the stability and adaptability (HMRPGV) and adaptability (RPGV), the cultivars CA 222, STAM 42 and ISA-205 were superiors.

Therefore, cultivars CA 222, STAM 42 will be the most recommended for farmers in cotton-growing regions and for a cotton breeding program of Mozambique.

Conflict of Interests

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

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Table 5. Adaptability of genotypic values (RPGV and RPGV*GM), stability and adaptability of genotypic values (HMRPGV and HMRPGV*GM) for cultivars evaluated in 19 environments from 2003/2004 to 2009/2010 growing season.

Cultivar	Genotypic value			
	RPGV	RPGV*GM	HMRPGV	HMRPGV*GM
CA-222	1.03	1,568.46	1.02	1,562.43
STAM 42	1.02	1,561.87	1.02	1,558.81
ISA 205	1.02	1,561.39	1.02	1,556.90
REMU 40	1.02	1,556.23	1.02	1,551.95
RMA 12-43	1.00	1,526.87	0.99	1,517.62
ALBAR SZ9314	0.99	1,520.04	0.99	1,513.91
ALBAR FQ902	0.99	1,517.89	0.99	1,511.97
CA 324	0.99	1,516.96	0.99	1,509.30
ALBAR BC853	0.93	1,428.64	0.93	1,423.08

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REFERENCES

Annicchiarico P (1992). Cultivar adaptation and recommendation from alfafa trials in Northern Italy. J. Gen. Plant Breeding, 46:269-278.

Benbouza H, Lacape JM, Jacquemin JM, Courtois B, Diouf FBH, Sarr D, Konan N, Baudoin JP, Mergeai G (2010). Introgression of the low gossypol seed and high-gossypol plant trait in upland cotton: analysis of [(*Gossypium hirsutum L. x G. raimondii*)2 x *G. sturtianum*] tri-specific hybrid and selected derivatives using mapped SSRs. Mol. Breed. 25:273-286.

Bias C, Donovan C (2003). Gaps and opportunities for agricultural sector development in Mozambique. Research Report, 54, MADER, Maputo p.72.

Bowman DT (2001). Common use of the CV: a statistical aberration in crop performance trials. The J. Cotton Sci. 5:137-141.

Brubaker CL, Wendel JF (1994). Reevaluating the origin of domesticated cotton (*Gossypium hirsutum L.*; Malvaceae) using nuclear restriction fragment length polymorphism (RFLPs). Am. J. Bot. 81:1309-1326.

Campbell BT, Jones MA (2005) Assessment of genotype x environment interactions for yield and fiber quality in cotton performance trials. Euphytica, 144: 69-78.

Carvalho P (1996). Manual of cotton. Ministry of Science and Technology, Institute of Tropical Science of Lisboa, p.282.

Contreras S, Krarup C (2000). Genotype and enviroment Interation of five cultivar (*Asparragus officinalis* L.). Ciencia and Agrarian Res. 27:133-139.

Cronn R, Wendel JF (2004). Cryptic trysts, genomic mergers, and plant speciation. New Phytologist. 161:133-142.

Cruz CD (2005). Principles of genetics quantitative. Viçosa: UFV, p.394.

Cruz CD, Carneiro PCS (2006). Biometric models applied to genetics and breeding. Viçosa: UFV, p.585.

Fengguo S, Xiangyun Z, Junlan L, Baosheng G, Junyi G, Yongzeng L, Shulong J, Zhinhong L, Yancai Z, Weiming L, Shezeng L, Wenlian P, Li Z, Chaojiang W, Senmao G (2007). Training course on cotton breeding and management technology. Shijiazhuan, China p. 231. Training course on cotton breeding and management technology. Shijiazhuan, China p.231.

Henderson CR (1975). Best linear estimation and prediction under a selection model. Biometrics 31: 423-447.

IAM [Cotton Institute of Mozambique] (2009). Report of the situation of Mozambican cotton sub-sector. Maputo p. 36.

INIA [National Institute of Agronomy Research] (2000). Agroecologic-Regions of Mozambique. Map, INIA, Maputo p. 1.

Khadi BM, Santhy V, Yadav MS (2010). Cotton: an introduction. Biotechnol. Agric. For. 65:1-14.

Köppen W (1948). Climatology: Studies of the climes´of the land. México: Fondo de Cultura Economica. p. 478.

Levene H (1960). Contributions to probability and statistics. Stanford, Stanford University Press, CA. pp. 278–292.

Lin CS, Binns MR (1988). A superiority measure of cultivar performance for cultivar x location data. Can. J. Plant Sci. 68:193-198.

Littell RC, Millken GA, Stroup WW, Wolfing RD, Schabenberger O (2006). SAS for mixed models. 2 ed. Cary: SAS Institute, p. 813.

MAE [Ministery of State Administration] (2005d). Profile of the distrital performance: Erati District. Province of Nampula. Maputo p.54.

MAE [Ministry of State Administration] (2005a). Profile of distrital performance: District of Meconta. Province of Nampula. Maputo p.45.

MAE [Ministry of State Administration] (2005b). Profile of distrital performance. District of Montepuez, Province of Cabo Delgado. Maputo p.51.

MAE [Ministry of State Administration] (2005c). Profile of the distrital performance: District of Morrumbala. Provínce fo Zambézia. Maputo p.52.

Maleia MP, Vidigal Filho PS, Kvitschal MV, Vidigal MCG (2010). Stability and adaptability of commercial cotton cultivars (*Gossypium hirsutum L. race latifolium H.*) in Mozambique. Afr. J. Agric. Res. 5:539-550.

Mendes FF, Guimarães LJM, Sousa JC, Guimarães PFO, Pacheco CAP, Machado JRA, Meireles WF, Silva AR, Parentoni SN (2012). Adaptability and stability of maize varieties using mixed models. Crop Breeding and Appl. Biotechnol. 12:111-117.

Mora F, Pupim-Junior O, Scapim CA (2007). Prediction of the cotton cultivar effect in the presence of genotype x environment interaction. Sci. Agrarian Res, 34:13-21.

Naveed M, Nadeem M, Islam M (2007). AMMI analysis of some upland cotton genotypes for yields stability in different millieus. World J. Agric. Sci. 3:39-43.

Oliveira RA, Rezende MDV, Daros EJ, Bespalhok FJC, Zambon JLC, Ido OT, Weber H, Koehler HS (2005). Genotypic evaluation and selection for sugarcane clones in three environments in State of Paraná. Crop Breeding and Appl. Biotechnol. 5:426-434.

Park YH, Alabady MS, Ulloa M, Sickler B, Wilkins TA, YU J, Stelly DM, Koyel RJ, El-Shihy OM, Cantrell RG (2005). Genetic mapping of new cotton fiber loci using EST-derived microsatellites in an interspecific recombinant inbred line cotton population. Molecular Genetics and Genomics 274: 428-431.

Piepho HP, Möhring J, Melchinger AE, Büchse A (2008). BLUP for phenotypic selection in plant breeding and variety testing. Euphytica, 161:209–228.

Piepho HP, Möhring J (2006). Selection in cultivar Trials - is It Ignorable? Crop Science 46:192-201.

Resende MDV (2002). Software Selegen–REML/BLUP. Curitiba: Embrapa Florestas, p.67.

Resende MDV (2007). Mathematics and estatistic in the experimental analysis and in genetic breeding. Colombo: Embrapa Forest, p.362.

SAS Institute Inc (2009). Software SAS 9.2. Cary, NC: SAS Institute Inc.
Shapiro M, Wilk B (1965). An analysis of variance test for normality (Complete Samples). Biometrika, 52:591-611.
Silva GO, Carvalho ADF, Vieira JV, Fritsche-Neto R (2012). Adaptability and stability of the carrot population. Horticultura Brasileira, 30:80-83.
Wallace TP, Bowman D, Campbell BT, Chee P, Gutierrez OA, Kohel RJ, Mccarty J, Myers G, Percy R, Robinson F, Smith W, Stelly DM, Stewart JM, Thaxton P, Ulloa M, Weaver DB (2008). Status of the USA cotton germplasm collection and crop vulnerability. Genet. Res. Crop Evol. 56:507-532.
Zhang HB, Li YN, Wang BH, Chee PW (2008). Recent advances in cotton genomics. Int. J. Plant Genome. p.20.

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Influence of processing methods on mycoflora changes during storage of raw and processed Atlantic horse mackerel (*Trachurus trachurus*)

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Study assessed the influence of processing on mycoflora of kote fillet, skin, head and bones (SHB) during storage for 28 days at ambient temperature of (32 ± 2°C). Fish samples were prepared by smoking (wood and coal) and poaching using standard methods. Fungi associated with raw and processed fillets and SHBs included the species of *Absidia glaucus*. *Absidia, Aspergillus flavus, Aspergillus niger, Aureobasidium* sp., *Candida tropicalis, Candida krusei, Fusarium* spp., *Rhizopus* sp., and *Penicillium expansum*. The various fungi was isolated using the direct plating and dilution plate methods indicated that wood smoke processing method had the highest ($p<0.001$) amount of mycoflora, which was followed by the coal smoke and poaching method. Also the SHB samples (fillet and SHB) had markedly low ($p<0.05$) fungal count than in the fillet. Thus, the SHB showed great promise in having lower ($p<0.01$) mycoflora which could be gathered and utilized at little costs for human food and animal feed, invariably reducing costs of feeds due to highly priced amount of casein, soybean meal and groundnut cake.

Key words: *Trachurus trachurus*, temperature, smoked fish and mycoflora.

INTRODUCTION

Fish is highly perishable but very important food stuff, due to its high levels of protein and polyunsaturated fatty acids as well as its affordability by the masses compared with beef. One of such species is the Atlantic horse mackerel (*Trachurus trachurus*), a medium-fat fish species abundant in the North-east Atlantic (Zimmermann and Hammer, 1999; Adeyemi et al., 2013). Nonetheless, chemical breakdown of protein, fat and water contents contribute to quick spoilage of fish (Adeyemi et al., 2013). Therefore various fish processing methods are used, to discourage/reduce the growth of spoilage organisms (Fayemi, 1999; Adetunji et al., 2007; Fagbohun et al., 2010) and increase the shelf-life of the stored product. Fish processing methods, like

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salting/brining, poaching, boiling, drying and smoking have been used for decades, this is because they allow for better preservation and storage as well as increase fish availability to the consumers (Egbal et al., 2010). More importantly is the skin, head and bone (SHB) of these processed fish could be gathered and utilized as a form of protein concentrate at little costs thus reducing costs of animal feeds due to highly priced casein, fish meal, soybean meal and groundnut cake (GNC) (Adeyemi, 2013).

Fungi dominate the micro flora of stored products, due to their ability to grow at low water content (Deible and Swamson, 2001). Mycoflora utilizes the nutrient contents of dry edible products, thus decreasing the value of food materials. Based on relative humidity field fungi attack developing and matured seeds in the field, while storage fungi are predominantly species of *Aspergillus* and *Penicillum* which attack stored products (Christensen, 1957). It is therefore important to know the quality of mycoflora of poached, coal and wood smoked *kote* and the cut off point of dried *kote* stored at ambient temperature (32 ± 2°C). Therefore, the objective of this work was to determine the effect of processing on the mycoflora of *Trachurus trachurus* fillet, skin, head and bones (SHB) during storage for 28 days at ambient temperature (32 ± 2°C).

MATERIALS AND METHODS

Collection of samples

Sample preparation and processing

A total of 20 kg (approximately 100 fish) of horse mackerel was collected from two popular major cold fish distributors (Asake and Heritage fisheries) in Ipata market, Ilorin, Nigeria. The mean length and weight of the fish was 30.52 ± 0.22 cm and 197.66 ± 3.67 g, respectively. *T. trachurus* was prepared using handling process that is, thoroughly washed, eviscerated and cooked by poaching and smoking using firewood (*A. seyal* and *C. lemon*) and charcoal. The processing methods were grouped into four (WSK: wood smoked kote; CSK: charcoal smoked kote; SK: poached kote; RK: raw kote).

Processing and packing of samples

A portion of the fish was poached in water at 60°C for 15 min and the remaining portion was smoked using either charcoal or firewood in a conventional smoke kiln as described by FAO/WHO/UN (2007). The fish smoking kiln was operated by first loading firewood into the heat chamber, preheated for 20 min and closed for 30 min to allow the smoking to take place after which fish samples were loaded into the central chamber. Fish was smoked at 80°C for 4 h; temperature was later increased to 105°C for 2 h and then returned to 80°C until the fish was properly smoked. The smoking time, temperature and ambient conditions were monitored using a thermometer during the smoking operation. Smoking was terminated when fish was properly dried after 8 h. The smoked fish were place in cane woven basket to cool off, after which portions of the processed fish were packaged in an insect free labelled transparent polythene bags and kept in the laboratory (32 ± 2°C).

Organoleptic test

A total of 10 member's panel evaluated the quality of the raw and processed products through sensory evaluation. Score sheet of sensory evaluation used in study was based on the method described by Standard National Indonesia, (1991). Sensory assessment was conducted via categorical ranking methods as described by DOCE (1989) and Eyo (2001). Four categories were ranked: highest quality, good quality, fair quality, and rejectable quality. The sensory assessment of the skin, eyes, gills, flesh odour, consistency, flavour, texture, colour and flesh appearance of the fish samples were also considered and scores among panelists were collected, statistically analyzed and expressed as the mean ± standard error (S.E.) (n=10), the significant differences between means were compared amongst the different processing methods using the least significant difference test after ANOVA for one-way classified data (Duncan, 1995). This was done to determine the taste, odour, texture and general appearance for the raw, poached and smoked *T. trachurus* samples. Products were scored on a scale of 10 - Excellent, 8 - Very Good, 6 - Good, 4 - Fairly good, 2 – Poor and 0 - extremely poor.

Analytical method

Cooking process was done without adding any ingredient. After poaching and smoke processes, a known portion of each fish species was oven dried to constant weight at 60°C, and the flesh of each fish was separated from its bones, skin and head. The skin, head and bones were collectively homogenized while the fillet alone was homogenized using a kitchen blender and stored on the shelf at ambient temperature for 28 days. After which the samples were examined for changes in the mycoflora periodically on day; 0, 3, 7, 14, 21 and 28, respectively.

Culture media

The media used in this study was nutrient agar. The media were prepared according to the manufacturer's specification. These media were sterilized in an autoclave at 121^0C for 15minutes.

Isolation of micro-organism

One gram of each sample (raw and processed kote fillet and SHB) was serially diluted, 1 ml of an appropriate dilution was inoculated on nutrient agar plates and the plates were incubated for 24 h at 30°C. After 24 h sterile wire loop was used to pick the isolate from the plate and was streaked on a freshly prepared sterile nutrient agar and MRS agar plates, then incubate for 24 h at 30°C in order to get pure cultures. The routine laboratory method of Cruickshank et al. (1975) as modified by Alexopoulous et al., (1996) was used to characterize different isolates. The isolates were identified using their macroscopic, cultural, physiological and biochemical characteristics.

Direct plating method

From the ambient stored processed *kote* samples, 10 g were examined randomly for external mouldness. They were surfaced sterilized with ethanol and later washed with sterile distilled water. Using a sterile dissecting forceps, the surface of the stored sun dried plantain chips were scrapped and were aseptically plated on potato dextrose agar (PDA) plate and incubated at room temperature for 5-7 days as described by Amusa (2001). The fungi cultures were further subcultured until pure colonies were obtained

Table 1. Fungi isolated from raw fillet and SHB stored at ambient temperature (32 ±2^{0}C) using different isolating methods.

Fungal		*A. niger*	*A. flaus*	*A. fumigatus*	*Mucor*	*Fusarium*	*Rizopus*	*Penicillum expansum*	*Aureobasidum*	*Candida tropicalis*	*C. krusei*	*Absidia*	*A. glaucus*
Dilution		**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**
FILLET	0	- -	- -	- -	+ -	- -	- -	- -	- -	- -	- -	- -	- -
	3	- -	- -	- -	+ -	- -	- -	- -	- -	- -	- -	- -	- -
	7	+ -	- -	- -	-	+ -	- -	- -	- -	+ -	- -	- -	- -
	14	+ -	- -	- -	+ -	+ -	- -	- +	- -	+ -	- -	- -	- -
	21	- -	- -	- -	+ -	+ +	+ -	- -	- -	-	- -	- -	- -
	28	- -	- -	- -	- -	+ -	- -	- -	- -	+ -	- -	- -	- -
		A B	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**
SHB	0	- -	- -	- -	- -	- -	- -	- -	- -	- +	- -	- -	- -
	3	- -	- -	- -	+ +	- -	- -	- -	- -	- +	- -	- -	- -
	7	- -	- -	- -	+ -	- -	- -	- -	- -	+ -	- -	- -	- -
	14	- -	- -	- -	+ -	- -	- -	- -	- -	+ -	- -	+ -	- -
	21	- -	- -	- -	+ -	- -	+ -	- -	- -	- -	- -	- +	- -
	28	- -	- -	- -	+ -	- -	- -	- -	- -	+ -	- -	- -	- -

A = Direct plating method B = Dilution plate method + = present (isolated) - = absent (not isolated). SHB= Skin head and bone.

by successive hyphae tip transfer (Egbebi et al., 2007). The cultures were examined under the microscope for fruiting bodies, hyphae to determine the common fungi present.

Dilution plate methods

This method was used to determine the type of fungi present in the ambient stored processed *kote* samples. About one gram of the sample was sterilized with ethanol and grinded with 10 ml of sterile distilled water. This was shaken thoroughly and 1ml of suspension was pipetted into a sterile test tube containing 9ml of distilled water. This was thoroughly mixed together. The sample was serially diluted and 1 ml each of aliquots of 10^{-4} and 10^{-5} were added to molten PDA plates. The plates were swirled gently to obtain thorough mixing and were allowed to solidify and incubated at room temperature for 5 - 7 days. The fungal colonies were counted every 24 h. Successive hyphae tip were transferred until pure cultures of each of fungus was obtained.

Identification of mycoflora

The associated fungi were identified by their cultural and morphological features (Alexopoulous et al., 1996). The isolates were examined under bright daylight for the colour of the culture and further examination was carried out.

RESULTS AND DISCUSSION

The microorganisms isolated from the raw, coal and wood smoked as well as poached (fillet and SHB) samples using different methods are represented in Tables 1 to 4 respectively. The scores done according to the Standard National Indonesia (1991) scheme for each parameter in the raw and processed samples are presented in Tables 5 and 6, respectively. A total of fungus were isolated from ambient (32 ± 2°C) stored raw and processed fillet and SHB, based on their cultural and morphological characteristics. The fungi include**:** *A. niger, A. flavus, A. fumigatus, Mucor, Fusarium*, *Rizopus, Penicillum expansum, Aureobasidum* spp*, Candida tropicalis, C. krusei, Absidia* and *A. glaucus* sp. The results indicated that *A. niger*, *A. flavus, A. fumigatus, Mucor*, *Fusarium*, *Penicillum expansum, Rizopu and Absidia)* were found in the coal smoked (fillet and SHB) stored samples; *A. niger, A. flavus, A. fumigatus, Mucor*, *Fusarium*, *Rizopus*., *Penicillum expansum, Aureobasidum, Candida tropicalis, C. krusei, Absidia* and *A. glaucus* spp) were found in the wood smoked stored. *A. niger, A. fumigatus, Mucor,*, *Penicillum expansum, Aureobasidum, Candida tropicalis,* and *Absidia*) in the poached

Table 2. Fungi isolated from coal smoked fillet and SHB stored at ambient temperature (32 ± 2°C) using different isolating methods.

Fungal		*A. niger*	*A. flaus*	*A. fumigatus*	*Mucor*	*Fusarium*	*Rizopus*	*Penicillum expansum*	*Aureobasidum*	*Candida tropicalis*	*C. krusei*	*Absidia*	*A. glaucus*
Dilution		**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**
FILLET	0	- +	- -	- -	+ -	- -	- -	- -	- -	- -	- -	- -	- -
	3	- -	- -	- -	+ -	- -	- -	- -	- -	- +	- -	- -	- -
	7	+ -	+ -	+ -	+ -	- -	- -	+ -	- -	- -	- -	- -	- -
	14	- -	- -	- -	+ -	+ -	- -	- +	- -	+ -	-	+ -	- -
	21	- -	- -	- -	+ -	+ -	- -	- -	- -	- -	- -	- -	- +
	28	- +	- -	- -	+ -	- -	- -	- +	- -	- -	- -	- -	- -
		A B	A B	A B	A B	A B	A B	A B	A B	A B	A B	A B	A B
SHB	0	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
	3	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	+ -	- -
	7	+ -	- -	- -	- -	+ -	- -	- -	- -	+ +	- -	+ -	- -
	14	+ -	- -	- -	- -	- -	- -	- -	- -	+ -	- -	- -	- -
	21	+ -	- -	- -	+ -	+ -	- -	+ -	- -	+ -	- -	- -	- -
	28	- -	- -	- -	+ -	- -	- -	- -	- -	- -	- -	- -	- -

A = Direct plating method, B = Dilution plate method, + = present (isolated), - = absent (not isolated), SHB= Skin head and bone.

Table 3. Fungi isolated from wood smoked fillet and SHB stored at ambient temperature (32 ±2°C) using different.

Fungal spp		*A. niger*	*A. flaus*	*A. fumigatus*	*Mucor Spp*	*Fusarium Spp*	*Rizopus*	*Penicillum expansum*	*Aureobasidum spp*	*Candida tropicalis*	*C. krusei*	*Absidia*	*A. glaucus*	*A. terreus*	*P. chanlybeum*
Dilution		***AB***	***AB***	***AB***	***AB***	***AB***	***AB***	***AB***	***AB***	***AB***	***AB***	***AB***	***AB***	***AB***	***AB***
FILLET	0	- -	- -	- -	+ -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
	3	- -	- -	- -	+ -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
	7	- -	+ -	- -	-	+ -	- -	- -	- -	+ -	- -	- -	+ -	- +	-
	14	+ -	- -	- -	+ -	+ -	- -	- -	- -	+ -	- -	- -	- -	- -	- -
	21	- -	- -	- -	+ +	+ -	+ -	- -	- -	- -	- -	- -	- -	- -	- -
	28	+ -	- -	+ -	+ -	+ -	- -	- -	- -	- -	- -	+ -	- -	- -	+ -
		A B	*AB*	*AB*	*AB*	*AB*	*AB*	*AB*	*AB*	*AB*	*AB*	*AB*	*AB*	*AB*	*AB*
HEAD	0	- -	- -	- -	+ -	+ -	- -	- -	- -	- +	- -	- -	- -	- -	- -
	3	- -	- -	- -	+ -	- -	- -	- -	- -	- +	- -	- -	- -	- -	- -
	7	- -	- -	- -	+ -	+ -	+ -	- -	- -	- -	- -	- -	- -	- -	- -
	14	- -	- -	- -	+ -	+ -	- -	- -	- -	+ +	- -	- -	- -	- -	- -
	21	- -	- -	- -	+ +	+ -	+ +	- -	- -	- -	- -	- -	- -	- -	- -
	28	- -	- -	- -	+ -	+ -	- -	- -	- -	+ -	+ -	+ -	- -	- -	- -

Table 4. Fungi isolated from poached smoked fillet and SHB stored at ambient temperature (32 ± 2°C) using different isolating methods

Fungal		*A. niger*	*A. flaus*	*A. fumigatus*	*Mucor*	*Fusarium .*	*Rizopus*	*Penicillum expansum*	*Aureobasidum*	*Candida tropicalis*	*C. krusei*	*Absidia*	*A. glaucus*	*Penicillum*
Dilution		**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**	**A B**
FILLET	0	- -	- -	- -	+ -	- -	- -	- -	- +	- -	- -	- -	- -	- -
	3	- -	- -	- -	+ -	- -	- -	- -	- -	- -	- -	- -	- -	- -
	7	+ -	- -	- -	+ -	+ -	- -	- -	- -	- -	- -	- -	- -	- -
	14	+ -	- -	- -	+ -	+ -	- -	- -	- -	+ -	- -	+ -	- -	- -
	21	- -	- -	- -	- -	- +	- -	- -	- -	- -	- -	- -	- -	- -
	28	- -	- -	- -	- -	+ -	- -	- -	- -	- -	- -	- -	- -	- +
		A B	A B	A B	A B	A B	A B	A B	A B	A B	A B	A B	A B	A B
HEAD	0	- -	- -	- -	- -	- -	- -	- -	- +	- -	- -	- -	- -	- -
	3	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
	7	- -	- -	- -	- -	+ -	- -	-	- -	- -	- -	+ -	- -	- -
	14	+ -	- -	- -	- -	- -	- -	+ -	- -	- -	- -	- -	- -	- -
	21	+ -	- -	- +	+ -	+ -	- -	- -	- +	- -	- -	- -	- -	- -
	28	- -	- -	- -	+ -	- -	- -	- -	- -	- -	- -	- -	- -	- +

A = Direct plating method, B = Dilution plate method, + = present (isolated), - = absent (not isolated). SHB= Skin head and bone.

Table 5. Showing Result of Sensory Evaluation of Processed *T. trachurus*.*

Parameter	CSK	WSK	PK
Odour	9.20 ± 0.32^{a}	8.80 ± 0.32^{a}	8.72 ± 0.55^{a}
Flavour/taste	9.20 ± 0.32^{a}	9.80 ± 0.20^{a}	8.00 ± 0.54^{b}
Texture	8.00 ± 0.79^{a}	8.20 ± 0.62^{a}	7.09 ± 0.62^{b}
Colour	9.60 ± 0.27^{a}	9.60 ± 0.40^{a}	7.27 ± 0.82^{b}

*Data= Mean ± SEM, n=10. Values with different superscripts along a row are significantly different ($P < 0.05$). CSK: Charcoal smoked *Kote*; WSK: Wood smoke *Kote*; PK: Poached *Kote*

samples compared to raw stored samples that had only fungi species (that is, *A. niger, Mucor*, *Fusarium, Rizopus, Penicillum expansum, Candida tropicalis,* and *Absidia*). Species of *Aspergillus, Mucor, Fusarium, Rhizopus,* and *Penicillum* fungi are known to be surface contaminant of most food products that induces decay. In this study the increase in quality of fungi in all isolates was similar to the report of Fagbohun et al. (2010), and Oladipo and Bankole (2013) but with the report of Ogundana et al. (1970) a decrease in fungi quantity in stored products was noticed. The fungi were likely to originate mainly from contamination from air and fish handling during processing, which was

Table 6. Showing Result of Sensory Evaluation of Raw *T. trachurus**

Parameter	Eye	Gill	Skin colour	Flesh texture
Rk	6.40 ±0.97	8.00±0.42	8.80 ±0.44	9.20±0.44
GRADE	2.60±0.85	1.70±0.26	1.70±0.26	1.70±0.26

*Data= Mean ± SEM, n=10. RK values were graded as described by Baremo de Classification de Frescura, (1989) & Eyo, (2001) (See Appendix I). Rk: Raw *kote*

detected from day 0 of storage. This fungus have been reported in immunosuppressed hosts such as in AIDS patients, non-AIDS patients with hematological malignancies and those receiving antifungal antibiotics that could alter the microbiota of human (Selik et al., 1997). *Penicillium* infections results in keratitis, endophtalmitis, otomycosis, necrotizing esophagitis, pneumonia, endocarditis, peritonitis, urinary tract infections, mucocutaneous, genitourinary, gastrointestinal, pulmonary and disseminated infections like the clinical features (Lueg et al., 1996; Mitchell et al., 1996; Kontogiorgi et al., 2007). Three species were isolated and identified from sundried plantain chips.

Aspergillus spp are common mould living in soil, hay etc. and the second most commonly recovered fungus in opportunistic mycoses. By transplantation, extensive use of immunosuppressive drugs which include corticosteroids predisposes human to *Aspergillus* infections (Douglas, 2007). The clinical features of *Fusarium* infections include keratitis, endophthalmitis, otitis media, onychomycosis, cutaneous pulmonary infections, endocarditis and fungemia (Lueg et al., 1996). Proper heating of food, elimination of infected and suspected food by *Fusarium* spp are the major preventive measures (Odds et al., 1998). *Rhizopus* spp and *C. krusei* were the least frequently encountered fungi in this study. The isolation was made at the 2nd week of storage.

Kontogiorgi et al. (2007) reported *Rhizopus* to causs rhinocerebral mucormycosis, mucocutaneous, genitourinary, gastrointestinal, pulmonary and disseminated infections. It is also responsible for the damage of blood vessels and nerves. Vascular invasion by *Rhizopus* causes necrosis of the infected tissue. Treatment of *Rhizopus* infections remains difficult due to its property to invade vascular tissues, infarction of the infected tissue is common and mortality rates are very high *Rhizopus* infections can be prevented by avoiding contact with contaminated object as well as maintaining a proper hygiene (Welsh and Kaplan, 1998).

Data obtained from sensory evaluation via categorical ranking method (Eyo, 2001), revealed that both the raw and processed fillet and SHB parts were in superior ($p < 0.05$) quality that warrant general acceptance (Tables 5 and 6). In addition, because of peroxidative damage to cellular membranes nutritional muscular dystrophy, fatty liver degeneration, anaemia, exudative diathesis, erythrocyte haemolysis, haemorrhages and depigmentation often observed in fish deficient of vitamin E (He and Lawrence, 1993; Mehrad et al., 2012); this was not the case in present study for both raw and processed (fillet and SHB) samples. Although PK was lowest in odour (8.72 ± 0.55), flavour (8.00 ± 0.54), texture (7.09 ± 0.62) and colour (7.27 ± 0.82), this values were still above the average score of 5 points for each fish product out of a maximum of 10 points, hence confirming that PK was also significantly ($p < 0.05$) acceptable for human food. Lastly, the average values of 6.40 ± 0.97 for eye, 8.00 ± 0.42 for gills, 8.80 ± 0.44 for skin colour and 9.20 ± 0.44 for flesh texture of indicated raw samples were of good quality and significantly high ($p < 0.05$) organoleptic acceptance. Nonetheless handling and processing of fish products, apart from good hygiene caution must be taken to reduce contamination by pathogens. This is because the isolated fungi can degrade both fillet and SHB as substrate, and pose a threat to the consumers by either infecting them or elaborating metabolites that can affect organs of the body.

Conclusion

Present study provided evidence for pathogenic fungi to enter, survive and grow within locally processed fish (kote fillet and SHB) samples. The samples subjected to the poaching method had markedly the least ($p<0.001$) presence followed by the charcoal and wood smoking method. The SHB samples also recorded significantly ($p<0.05$) low amounts of presence in all the (raw and processed) samples compared to the fillet (raw and processed). Since, the SHB showed great promise in longer ($p<0.01$) keeping quality at ambient temperature than the fillet. Results suggests that foods processed from the SHB could serve as healthy low cost food that would help increase the importance of wastes which if left uncared may cause pollution to the environment. Due to the high cost of protein concentrate and fish meal used in animal feeds, the SHB could be converted to nourishable feeds.

REFERENCES

Adetunji VO, Alonge R, Singh K, Chen J (2007). Production of wara, a West African soft cheese using lemon juice as a coagulant. J. Food Sci. Technol. 41(2): 331-336.

Adeyemi OT (2013). Effect of Processing on the Nutrient and Anti-Nutrient Composition of Atlantic Horse Mackerel (*Trachurus trachurus)* In Weaned Male Wistar Rats. Ph.D. Thesis. Babcock University Ilisan Remo, Ogun State.

Adeyemi OT, Osilesi OO, Onajobi FD, Adebawo O, Afolayan AJ (2013). Effect of Processing on The Proximate and Mineral Compositions of *Trachurus trachurus*: A Fish Commonly Consumed in Nigeria. J. Emerging Trends i Engineering Appl. Sci. (JETEAS). 4(3):378-385.

Adeyemi OT, Osilesi OO, Onajobi FD, Adebawo O, Afolayan AJ (2013). Stability study of smoked fish, horse mackerel (*Trachurus trachurus*) by different methods and storage at room temperature. Afr. J. Biochem. Res. 7(6):98-106.

Alexopoulous CJ, Mims CW, Blackwell M (1996). Introductory Mycology. 4th edition. John Wiley & Sons, Inc., New York. pp. 127-171.

Amusa NA (2001). Fungi associated with yam chips in storage and the effect on the chips nutrients composition. Moor J. Agric. Res. 2:35-39.

Amusa NA, Kehinde IA, Ashaye OA (2002). Biodeterioration of bread fruit (*Artocarpus communis*) in storage and its effects on the nutrient composition. Afr. J. Biotechnol. 1(2): 57-60.

Christensen CM (1957). Deterioration of stored grains by fungi. Annu. Rev. Phytopath. 3:69-84.

Cruickshank R, Duguid JP, Marmion BP, Swainr HA (1975). Medical Microbiology, vol. 2, Practice of Medical Microbiology. Edinburgh, London and New York: Churchill Livingstone.

Deible KE, Swanson KMJ (2001). Cereal and cereal products. In F. P. O. Downes and K. Ito (eds). Compendium of Methods for the Microbiological Examination of Foods. Blackwell Pub. Co, London. pp. 98-102.

DOCE (1989). Baremo de Clasificacion de Frescura, In: Diario Official de las Comunidades Europeans nº L5/21, 07. 01. European Commission, Brussels. 5–6.

Douglas F (2007). Mycotic Infections. Del Med. J. 69:1-4.

Duncan DB (1955). Multiple Range and Multiple F-test. Biometrics 11:1 - 5.

Egbal O, Ahmed M, Ali E, Regiah A, Kalid HM, Taha A, Mahammed A (2010). Investigating the Quality Changes of Raw and Hot Smoked *Oreochromis niloticus* and *Clarias lazera*. Pak. J. Nutr. 9 (5): 481-484

Egbebi AO, Anibijuwon II, Fagbohun ED (2007). Fungi associated with dry cocoa beans during storage in Ekiti State, Nigeria. Pak. J. Nutr. In press.

Eyo AA (2001). Fish Processing Technology In The Tropics. University of Ilorin Press, Ilorin, Nigeria.

Fagbohun ED, Abegunde OK,David OM (2010). Nutritional and mycoflora changes during storage of plantain chips and the health implications. J. Agric. Biotechnol. Sustainable Dev. 2(4): 61-65.

FAO/WHO/UNU (2007). "Protein and Amino Acid Requirements In Human Nutrition": Report of a Joint WHO/FAO/UNU Expert Consultation. World Health Organ Tech Rep. Ser #: 935. Geneva, Switzerland: FAO/WHO/ UNU. WHO Press. 150:179-276.

Fayemi PO (1999). Nigerian vegetables. Heinemann Educational Books (Nigeria) Plc. pp.15-20.

He H, Lawrence AL (1993). Vitamin E Requirement of Penaeus vannemei. Aquaculture 118: 245-255.

Kontogiorgi M, Floros I, Koroneos A, Vamvonka C, Paniara O, Roussos C, Routi C (2007). Fatal post-truamatic zygomycosis in an immuno-competent young patient. J. Med. Microbiol. 56:1243-1245.

Lueg EA, Ballagh H, Forte J (1996). Analysis of the recent cluster of invasive fungal sinusitis at the Toronto Hospital for sick children. J. Otolaryngol. 25: 366-370.

Mehrad B, Hojatollah J, Mehdi MT (2012). Assessment of The Effects Of Dietary Vitamin E On Growth Performance And Reproduction of Zebra Fish, *Danio rerio* (Pisces, Cyprinidae). J. Oceanography Marine Sci. 3(1):1-7.

Mitchell SJ, Gray J, Morgan MEI, Hocking MD, Durbin GM (1996). Nosocomial infection with *Rhizopus microsporus* in preterm infants: association with wooden tongue depressors. Lancet 348: 441-443.

Odds FC, Van Gerven F, Epsinel-Ingroff A, Bartlett MS, Ghannoum MA, Lancaster MV, Pfaller MA, Rex JH, Rinaldi MG, Walsh TJ (1998). Evaluation of possible correlations between antifungal susceptibilitiesof filamentous fungi *in-vitro* and antifungal treatment outcomes in animal infection models. Antimicrob. Agents Chemother. 42: 282-288.

Ogundana SK, Naqui SH, Ekundayo JA (1970). Fungi associated with soft rot of yam (*Discorea* spp) in Nigeria. Trans. Br. Mycol. Soc. 54: 445-451.

Oladipo IC, Bankole SO (2013). Nutritional and microbial quality of fresh and dried *Clarias gariepinus* and *Oreochromis niloticus*. Int. J. Appl. Biochem. Biotechnol Res. (IJAMBR). 1: 1-6.

Selik RM, Karon JM, Ward JW (1997). Effect of the human immunodeficiency virus epidemic of mortality from opportunistic infections in the United States in 1983 Infect. Dis. 176: 632-636.

Standard National Indonesian (SNI) (1991). Chemical Analysis of Fisheries Product. National Standaritation Agency, Standar Nasional Indonesia, Analisa Kimiawi Produk-Produk Perikanan, Badan Standarisasi Nasional.

Welsh TS, Kaplan J (1998). The role of postmortem examination in medical education. Mayo Clin. Proc. 73: 802-805.

Zimmermann C, Hammer C (1999). On the Biology of the Horse Mackerel in North and North-East Atlantic. Inf. Fischwirtsch. Fischereiforsch 46:14-23.

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